การเพิ่มความเข้มข้นสุดท้ายของกรดแอลแล็กติกผ่านการหมักแบบเฟดแบตช์
โดย *Bacillus aerolacticus* BC-001

นางสาวอนัญพร วุฒิมงคลชัย

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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INCREASING FINAL CONCENTRATION OF L-LACTIC ACID VIA FED-BATCH FERMENTATION BY *Bacillus aerolacticus* BC-001

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A Thesis Submitted in Partial Fulfillment of the Requirements
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Thesis Title	INCREASING FINAL CONCENTRATION OF L-LACTIC ACID VIA FED-BATCH FERMENTATION BY *Bacillus aerolacticus* BC-001
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อนัญพร วุฒิมงคลชัย : การเพิ่มความเข้มข้นสุดท้ายของกรดแอลแล็กติกผ่านการหมักแบบเฟดแบตช์โดย *Bacillus aerolacticus* BC-001 (INCREASING FINAL CONCENTRATION OF L-LACTIC ACID VIA FED-BATCH FERMENTATION BY *Bacillus aerolacticus* BC-001) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: รศ. ดร. ณัฏฐา ทองจุล, 91 หน้า.

พอลิแล็กติกแอซิดเป็นที่ได้รับความสนใจอย่างมาก เนื่องจากสามารถใช้งานได้หลากหลาย แต่การแข่งขันด้านต้นทุนกับปิโตรเลียมพลาสติกนั้นยังเป็นปัญหาที่สำคัญในอุตสาหกรรมพลาสติกชีวภาพ ซึ่งวิธีการหนึ่งที่จะลดต้นทุนการผลิตพลาสติกชีวภาพที่สามารถทำได้คือการใช้วัตถุดิบที่มีต้นทุนต่ำ ดังนั้นการได้ผลิตภัณฑ์ความเข้มข้นสุดท้ายที่สูงจึงเป็นที่ต้องการในระดับอุตสาหกรรม อย่างไรก็ตามการหมักแบบแบตช์ที่ใช้ความเข้มข้นของสารตั้งต้นสูงมักจะเกิดปัญหาการให้อัตราผลผลิตต่อกลูโคสและอัตราการผลิตต่ำจากการยับยั้งของสารตั้งต้น ในงานวิจัยนี้ได้ทดสอบความสามารถของ *Bacillus aerolacticus* BC-001 ในการหมักน้ำตาลกลูโคสที่มีความเข้มข้นสูงถึง 200 กรัมต่อลิตร และศึกษาการหมักแบบเฟดแบตช์ทั้งในระดับขวดเขย่าและถังหมักแบบกวนขนาด 5 ลิตร ในการศึกษาระดับขวดเขย่าพบว่า *B. aerolacticus* BC-001 สามารถหมักน้ำตาลกลูโคสที่ความเข้มข้นสูงถึง 200 กรัมต่อลิตรได้ โดยยังให้อัตราผลผลิตต่อกลูโคสและอัตราการผลิตที่ใกล้เคียงกับการหมักด้วยน้ำตาลกลูโคส 100 กรัมต่อลิตร นอกจากนี้เมื่อทำการหมักแบบเฟดแบตช์ยังสามารถให้ความเข้มข้นสุดท้ายของกรดแล็กติกที่สูงขึ้นถึง 192 กรัมต่อลิตร ด้วยอัตราผลผลิตต่อกลูโคสที่ 0.90 กรัมต่อกรัม และอัตราการผลิตที่ 3.55 กรัมต่อลิตรต่อชั่วโมง เมื่อทำการหมักในถังหมัก 5 ลิตร โดยวิธีการหมักแบบแบตช์ด้วยสารละลายกลูโคสสามารถให้ความเข้มข้นสุดท้ายของกรดแล็กติก104.24 กรัมต่อลิตร ด้วยอัตราการผลิตและอัตราผลผลิตต่อกลูโคส 5.79 กรัมต่อลิตรต่อชั่วโมง และ 0.85 กรัมต่อกรัมตามลำดับ และเนื่องจากการเพิ่มความเข้มข้นของกลูโคสสูงขึ้นที่ 125-200 กรัมต่อลิตรส่งผลให้เกิดการยับยั้งของสารตั้งต้น จึงมีการใช้การหมักแบบเฟดแบตช์เข้ามาเพื่อแก้ปัญหาดังกล่าว โดยวิธีที่มีประสิทธิภาพมากที่สุดคือการหมักแบบเฟดแบตช์ที่แบ่งเติมกลูโคสเป็นช่วงๆ ซึ่งให้ความเข้มข้นสุดท้ายของกรดแล็กติก 145.08 กรัมต่อลิตร อัตราการผลิต 3.72 กรัมต่อลิตรต่อชั่วโมง และอัตราผลผลิตต่อกลูโคส 0.95 กรัมต่อกรัม นอกจากนี้พบว่าเมื่อเติม $(NH4)_2SO_4$ ร่วมกับกลูโคสด้วยอัตราคาร์บอนต่อไนโตรเจนที่ 40 สามารถเพิ่มความเข้มข้นสุดท้ายของกรดแล็กติกสูงขึ้นถึง 160.84 กรัมต่อลิตร ด้วยอัตราการผลิต 3.58 กรัมต่อลิตรต่อชั่วโมง และให้อัตราผลผลิตต่อกลูโคสอยู่ที่ 1.02 กรัมต่อกรัม เมื่อทำการเปรียบเทียบกับการหมักแบบแบตช์แล้วจะพบว่าวิธีการนี้สามารถเพิ่มความเข้มข้นสุดท้ายของกรดแล็กติกได้ถึง 54.30 เปอร์เซ็นต์ เพิ่มอัตราผลผลิตต่อกลูโคส 11.76% และสามารถยืดอัตราการผลิตที่ 11.70 กรัมต่อลิตรต่อชั่วโมงไว้ได้ 18 ชั่วโมง

สาขาวิชา เทคโนโลยีชีวภาพ ลายมือชื่อนิสิต .....

ปีการศึกษา 2559 ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

5672220523 : MAJOR BIOTECHNOLOGY
KEYWORDS: L-LACTIC ACID, FED-BATCH FERMENTATION, GLUCOSE REPRESSION, POLYLACTIC ACID

ANUNYAPORN WUTTHIMONGKHOLCHAI: INCREASING FINAL CONCENTRATION OF L-LACTIC ACID VIA FED-BATCH FERMENTATION BY *Bacillus aerolacticus* BC-001. ADVISOR: ASSOC. PROF. NUTTHA THONGCHUL, Ph.D., 91 pp.

Polylactic acid (PLA) now gains many interests due to its versatile applications. Nonetheless, cost competitiveness with the existing petroleum based plastic is the key issue in biobased plastic industry. One plausible way to reduce production cost of biobased plastic can be accomplished by low cost feedstocks. Therefore, high final product titer at the end of batch run is necessary. However, batch fermentation using high substrate concentration usually suffers from low yield and productivity due to substrate repression. In this work, we tested for the ability of *Bacillus aerolacticus* BC-001 to ferment glucose at high concentration up to 200 g/L and study using fed-batch fermentation in both flask and 5 L stirred fermentation cultures. In flask, the results revealed that *B. aerolacticus* BC-001 was able to ferment glucose at 200 g/L at the close yield and productivity to those obtained from 100 g/L glucose. Moreover, final lactate titer be increased up to 192 g/L with the acceptable yield of 0.90 g/g and productivity of 3.55 g/L×h by intermittent fed-batch fermentation. In case of fermentation in 5 L fermentor, via batch fermentation with sole glucose solution, 104.24 g/L lactate titer with the productivity and yield of 5.79 g/L×h and 0.85 g/g respectively was obtained. When increasing the initial glucose concentration to 125-200 g/L, substrate repression was appeared. To solve this problem, different fed-batch fermentation were conducted. The most effective feeding program was intermittent fed-batch fermentation. The final lactate titer of 145.08 g/L with productivity of 3.72 g/L×h and yield of 0.95 g/g was obtained. Moreover, adding glucose solution supplemented with $(NH_4)_2SO_4$ at the C/N ratio of 40:1 was able to improve final lactate titer to 160.84 g/L with 3.58 g/L×h and 1.02 g/g. Therefore, compared with typical batch fermentation, intermittent fed-batch fermentation with glucose solution supplemented with $(NH_4)_2SO_4$ at the proper C/N ratio produced higher final lactate concentration for 54.30% with the increasing yield by 11.76%. Most importantly, this technique could prolong high production rate at 11.70 g/L×h until 18 h fermentation.

Field of Study: Biotechnology Student's Signature .....

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CHAPTER 1

INTRODUCTION

1.1 Background and Rationale

Nowadays, the plastic demand is significantly increased because plastics are substitute several metals in automobiles and digital devices due to their outstanding properties, e.g. light weight, low cost, biocompatibility, easy to fabricate, and easy to use. Although the demand is kept rising, commodity plastics are now mostly synthesized from petrobased feedstocks that is considered as non renewable and price instability. Importantly, most of commodity plastics used are partially degraded; therefore, the hugh consumption generates environmental impact (Goodall, 2011). Inspite of the prefound pollution problem, plastic consumption still cannot be prohibited because of its versatile applications. The environmental impact from petroleum based plastics can be lessened by making plastics become biodegradable. Bioplastic with its excellent properties including biodegradability, biocompatibility, and renewability becomes of interest (Müller et al., 2012).

Polylactic acid, synthesized via ring opening polymerization of an optically pure lactic acid, is one of the promising biodegradable plastics (Zhao et al., 2010). This biodegradable polymer can be either blended or copolymerized with other polymers so that the properties can be competitive with those in commodity plastics. Not only the properties that meets the consumer demands, cost competitiveness is also mandatory. To replace commodity plastic market, the production cost of polylactic acid should be

low or even lower than that of petroleum based plastic. To achieve that goal, lactic acid feedstocks must be supplied for polylactic acid production facility at low price with sufficiently high polymer grade quality.

Lactic acid naturally exists in 2 forms, i.e., L-lactic acid and D-lactic acid. L-lactic acid has been widely found from natural resources including fermented products and it has been extensively used in food and pharmaceutical applications. On the other hand, D-lactic acid is scarcely found in nature and it is considered as toxic to living organisms when exposed at high level (Reddy et al., 2008). To date, fermentation process, considered as green process compared with the chemical synthesis, is commonly used in commercial lactic acid production (Abdel-Rahman et al., 2013; Ren, 2011).

Commercial lactic acid product currently available in the market is sold in the racemate form with the approximate enantiomer percentage of 85% (L-lactate). Unlike other existing applications in food and pharmaceutical industries, lactic acid feedstocks entering polylactic acid production process is required to meet adequately high enantiomer percentage (>99.0 %ee) (John et al., 2009). Therefore, the commercial lactate product currently available in the market cannot be directly used for polylactic acid synthesis.

There have been many attempts to improve lactic acid production in term of polymer grade quality and the production cost. Process optimization together with process integration have been developed for improving both upstream fermentation process and downstream lactate recovery and purification. Furthermore, strain development for selection of the robust organisms has been extensively conducted using several genetic tools. Recently, metabolic engineering has been introduced to

obtain the evolved strain with all specified functions required in industrial fermentation. Many organisms can produce lactic acid. Lactic acid bacteria including *Bacilli*, *Lactobacilli*, *Streptococci*, and *Enterococci* are the typical lactic acid producing groups. Some yeasts and fungi can also produce lactic acid. Those include *Rhizopus* sp., *Mucor* sp., and *Monilia* sp., for instance. Bacterial fermentation usually yields high production rate while fungal fermentation provides advantages in term of high optical purity of lactic acid product suitable for polymer grade specification for polylactic acid synthesis. Nonetheless, running industrial fermentation using fungi experiences the change of morphology and difficulty in controlling process parameters as a result of failure to controla and maintain suitable morphology throughout the operation (Groot et al., 2010). Therefore, to date bacterial fermentation is still widely used in lactic acid production.

Batch process is commonly used in industrial lactic acid fermentation due to simple process design, easy operating procedure, and low risk of contamination. Since there is no inlet/outlet streams entering/discharging from the fermentor except gas inlet/outlet and base addition for pH control, contamination is easy to control in batch operation. However, this operation mode may be suffer from substrate repression, product inhibition, low yield, and low productivity at high initial substrate concentration to obtain high final product titer. To obtain high quantity of lactate product, a series of fermentors and a large downstream processing units are required. On the other hand, continuous operation reduces substrate repression and product inhibition but experiences high risk of contamination, change in production performance, and wash out during long term cultivation. Fed-batch operation is usually applied for optimizing the pros and cons of batch and continuous cultures. During fed-

batch operation, substrate is gradually fed into the fermentor; therefore, reducing substrate repression while the increasing liquid broth remained in the fermentor until the end of fermentation. By this, product concentration gradually increases as a result of dilution when substrate is fed into the fermentor; therefore, this can prolong the operating time, the evidence of product inhibition, and eventually the large amount of product mass obtained from the culture. Several feeding programs have been developed in fed-batch cultivation. Four distinctive programs include (1) pulse feeding or intermittent feeding when substrate is fed once at a time, (2) constant feed rate when substrate is fed at the constant flowrate, (3) exponential feed rate when substrate is fed according to the specific growth rate of the culture, and (4) constant residual substrate concentration where during the operation substrate is fed to compensate the consumption in order to maintain the concentration at the set point (Zhang et al., 2010).

The results reported in previous literatures confirm that fed-batch was successfully used in lactic acid fermentation. In 2006, Ding and Tan studied fed-batch fermentation of lactic acid using 4 different feeding programs mentioned above. They found that all 4 feeding programs gave higher lactic acid production than that obtained in batch culture (Ding and Tan, 2006). The results reported in Li et al. (2010) are consistent with those mentioned in Ding and Tan (2006). Higher final lactate titer was obtained in fed-batch operation compared with that in batch culture as the fermentation time was prolonged. While the productivity could be lower than batch operation when the feeding program was not suitable (Li et al., 2010). In 2012, Gao et al. applied intermittent feeding program in fed-batch lactic acid fermentation to reduce substrate repression (Gao et al., 2012). By this, the improved productivity and high lactate titer were obtained. As aforementioned, proper process optimization is required in fed-batch

operation in order to achieve high final titer while the product yield and fermentor productivity can be maintained as high as those obtained in batch operation.

Our in-house isolate, *Bacillus aerolacticus* BC-001 (accession number: NITE BP-01943) was proven for its ability to ferment lactic acid from glucose at the remarkably high lactate yield and productivity during batch cultivation. To further improve the performance of our isolate *B. aerolacticus* BC-001, fed-batch operation was applied in lactate fermentation. Several feeding programs have been tested for increasing the final lactate titer with high yield and productivity.

1.2 Research objectives and scope of work

To increase final lactate titer with high yield and productivity during lactate fermentation by *B. aerolacticus* BC-001 via fed-batch operation.

The optimized feeding program that gave the high final titer, yield, and productivity was determined. The compositions of feeding solution, glucose concentration to be fed, nitrogen sources to be supplemented, and the appropriate C/N ratio were varied.

1.3 Expected outcome

Via optimized fed-batch operation, sufficiently high final lactate titer suitable for downstream recovery process with the similarly high yield and productivity to those obtained in batch cultivation was obtained.

CHAPTER 2

THEORETICAL AND LITERATURE REVIEWS

2.1 Lactic acid

Lactic acid, chemically 2-hydroxypropionic acid or 2-hydroxypropanoic acid**,** CH_3-CHOHCOOH**,** is an organic acid widely distributed throughout nature discovered in 1780 by the Swedish chemist C.W. Scheele in sour milk. It was later first produced commercially in 1881 by Charles E. Avery in Massachusetts, USA. It exists naturally in two enantiomeric forms: D(−)- lactic acid and L(+)-lactic acid (Figure 2.1). Moreover, chemical synthesis results in a racemic mixture of two isomers.

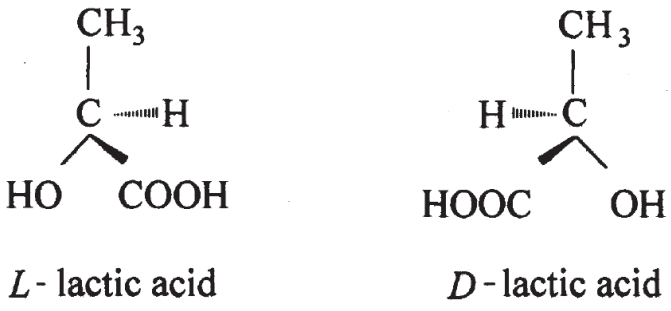


Figure 2.1 Isomer structure of Lactic acid

The difference between the two enantiomeric forms is an adjacent hydroxyl group at the chiral carbon atom. Due to this there exist some different properties such as boiling and melting points. High optical purity lactic acidis colorless, miscible with water or ethanol, acetone and ether. However, it is not soluble in chloroform, petroleum

ether or carbon disulfide. Other physical-chemical properties are summarized below in Table 2.1 (Castillo Martinez et al., 2013; Ren, 2011; Vaidya et al., 2005).

Table 2.1 Identification and physical and chemical properties

Parameter	
CAS number	D/L: 50-21-5 L: 79-33-4 D: 10326-41-7
EINECS number	200-018-0
H.S. code	2918.11
Formula	CH_3CH (OH) COOH
Molecular mass	90.08 g/mol
Specific gravity	1.2 g/mL
Melting point	L: 53 °C D: 53 °C D/L: 16.8 °C
Boiling point	122 °C (12 mmHg)
Flash point	112 °C
Physical state	Colorless to slinghtly yellow, syrupy liquid
Solubility in water	Miscible
Taste	Mild acid taste
Toxicity	Oral rat LD 50:3543 mg/kg
NFPA ratings	Health 3, Flammability 1, Reactivity 1
Stability	Stable under ordinary conditions

2.2 Application

Lactic acid has been classified by the Food and Drug Administration (US FDA) for use as a food additive, and it has been widely used in the food and pharmaceutical industries. Due to elevated levels of the D-isomer being harmful to humans, L-isomer is the preferred choice for food-related and pharmaceutical industries.Moreover, lactic acid is also applied in the chemical, pharmaceutical, plastics, cosmetics and medicine industries. (Datta et al., 1995).

2.2.1 Food and beverages industry

Lactic acid is widely applied in food products such as bread, meat, milk, beverages, desserts, among others. Lactic acid is used as an acidulant to increase acidity and to serve as a pH regulator and flavoring agent. Furthermore, due to the acidity being able to prevent the growth of pathogens, it helps in preservation. Moreover, many fermented foods naturally produce lactic acid such as yogurt, cheese, milk, soy sauce, wine, meat products, and pickled vegetables (Callewaert and De Vuyst, 2000).

2.2.2 Cosmetic industry

In the cosmetics industry, lactic acid is popularly known as alpha hydroxy acid or AHA. Lactic acid is used as a pH regulator, moisturizer and antimicrobial agent. It is a mixture of cream or lotion to hydrate and rejuvenate the skin. Additionally, by inhibition of the formation of tyrosinase, lactic acid is also used as a skin lightener.

2.2.3 Pharmaceutical industry

Lactic acid is used as a pH regulator by helping to adjust the pH. In addition, lactic acid can enhance drug solubility through use as an intermediary in assisting improvement of the drug combination. Moreover, lactic acid assists in preventing oxidation reactions in vitamin preparation, serves as metal sequestration agent, and a natural body constituent. Due to its descaling and antibacterial properties, lactic acid is also used in cleaning products. Furthermore, lactic acid derivatives are used to treat diseases such as calcium deficiency in bones and teeth, and anemia.

2.2.4 Polymer industry

Currently, lactic acid is used as a monomer of polylactic acid or PLA production, a biodegradable plastic that is produced as a substitute for petrochemicals plastic. PLA are classified into aliphatic polyester which high strength thermoplastic. These can be digested easily by hydrolysis of the ester bond (Abdel-Rahman et al., 2013; Vijayakumar et al., 2008).

2.3 Lactic acid production

Lactic acid can be produced by either chemical synthesis or microbial fermentation. Incidentally, 90 percent of lactic acid is produced frommicrobial fermentation. Chemical synthesis of lactic acid is mainly based on the hydrolysis of lactonitrile by strong acids, and this process yields a racemic mixture of the two isomers (John et al., 2007).

2.3.1 Chemical synthesis

Industrially, chemical synthesis process involves synthesis from lactonitrile. First, lactonitrile is produced by the reaction of hydrogen cyanide (HCN) and acetaldehyde occurring at high atmospheric pressures in the liquid phase. After that,purified lactonitrile is hydrolyzed to lactic acid by sulfuric acid or hydrochloric acid with lactic acid and ammonium salt being obtained. Before purification through distillation and hydrolysis by water, lactic acid is esterified by methanol to produce methyl lactate. This process explains the following reaction.

(a) Addition of Hydrogen Cyanide

$$CH_3CHO + HCN \xrightarrow{\text{catalyst}} CH_3CHOHCN$$

Acetaldehyde Hydrogen cyanide Lactonitrile

(b) Hydrolysis by H_2SO_4

$$CH_3CHOHCN + H_2O + 1/2H_2SO_4 \longrightarrow CH_3CHOHCOOH + 1/2(NH_4)_2SO_4$$

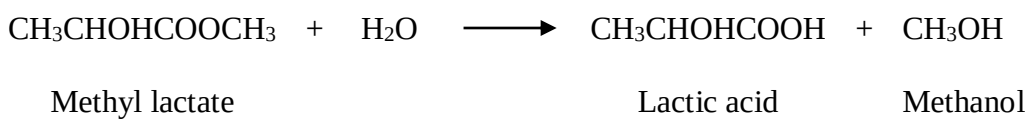
Lactonitrile Sulfuric acid Lactic acid Ammonium salt

(c) Esterification

$$CH_3CHOHCOOH + CH_3OH \longrightarrow CH_3CHOHCOOCH_3 + H_2O$$

Lactic acid Methanol Methyl lactate

(d) Hydrolysis by H_2O



However, chemical synthesis has some disadvantages including that the reaction has to occurat high atmospheric pressures. Additionally, chemical synthesis requires chemical derivatives from petrochemicals as raw materials whose prices are currently increasing and which are not environmentally friendly. Furthermore, the racemic mixture of the two isomers that is obtained cannot be used in the food and pharmaceutical industries due to the toxicity of D-lactic acid to the human body. Therefore, the fermentation process is preferable (Randhawa et al., 2012).

2.3.2 Microbial fermentation

Fermentation is a process found in animal cells and some bacteria in anaerobic conditions. This process converts substrate (sugars such as glucose, sucrose, and xylose) into energy and fermented products such as lactic acid, fumaric acid, ethanol, and acetic acid. Via fermentation, lactic acid can be produced by fungi or bacteria.

2.3.2.1 Fungal fermentation

Fungi are able to produce lactic acid from agricultural raw materials that are cheap and easy to find because of their ability to produce extracellular amylase to hydrolyze raw materials such as starch into sugar, a substrate of lactic acid production and cell growth. A widely found strain of fungi with the ability to produce lactic acid

is *Rhizopus sp.*The cost of fungal fermentation is high due to the requirement of a lot of oxygen for growth and lactic acid production. Moreover, fungal fermentation is heterofermentation which can produce lactic acid and by-products such as fumaric acid, alcohol, and carbon dioxide.

2.3.2.2 Bacterial fermentation

Lactic acid bacteria is fastidious; a wide range of growth factors including specific minerals, amino acids, vitamins, fatty acids, purines, and pyrimidines are required for their growth and biological activity. Additionally, lactic acid bacteria do not require a lot of oxygen as fungi and are easy to culture in a fermentor because the bacteria are single-celled. Because of this, the production cost is cheaper than fungal fermentation.

There are four steps of bacterial fermentation as follows:

(a) Fermentation and neutralization

$$C_6H_{12}O_6 + Ca(OH)_2 \xrightarrow{\text{fermentation}} 2(CH_3CHOHCOO^-)\,Ca^{2+} + 2H_2O$$

Carbohydrate — Calcium hydroxide — Calcium lactate

(b) Hydrolysis by H_2SO_4

$$2(CH_3CHOHCOO^-)\,Ca^{2+} + H_2SO_4 \longrightarrow 2CH_3CHOHCOOH + CaSO_4$$

Calcium lactate | Sulfuric acid | Lactic acid | Calcium sulfate

(c) Esterification

$$CH_3CHOHCOOH + CH_3OH \longrightarrow CH_3CHOHCOOCH_3 + H_2O$$

Lactic acid | Methanol | Methyl lactate

(d) Hydrolysis by H_2O

$$CH_3CHOHCOOCH_3 + H_2O \longrightarrow CH_3CHOHCOOH + CH_3OH$$

Methyl lactate | Lactic acid | Methanol

Lactic acid bacteria are divided according to fermentation patterns into the two groups homofermentative and heterofermentative.

Homofermentative is the group of bacteria that ferments more than 85% glucose with lactic acid as the primary product through the Embden-Meyerhof-Parnas (EMP) glycolytic pathway. They convert 1 mol of glucose to 2 mol of lactic acid and 2 mol ATP. Homofermentative lactic acid bacteria include*Lactobacillus acidophilus, L. bulgaricus, Lactococcus* spp., and *Streptococcus* spp.

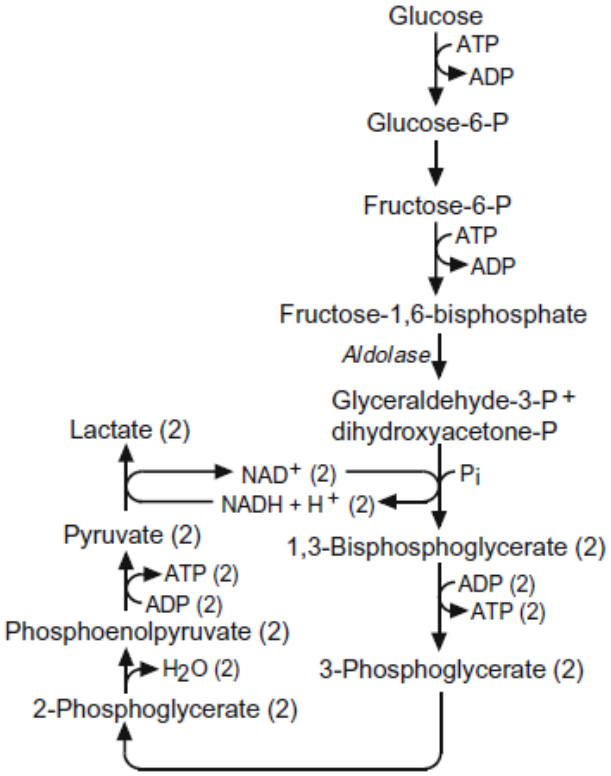


Figure 2.2 Homofermentative metabolism pathway of lactic acid bacteria

Heterofermentative is a group of bacteria that ferment glucose into both products including lactic acid (50%) and ethanol/acetic acid and carbon dioxide (CO_2). Thus, heterofermentative bacteria can be tested by the detection of gas (e.g., CO_2). Heterofermentative lactic acid bacteria convert 1 mol of glucose to 1 mol of lactic acid, 1 mol of ethanol/acetic acid, and 1 mol of CO_2. Bacteria in this group include *Lactobacillus brevis, L. fermentum,* and *Leuconostoc* spp.

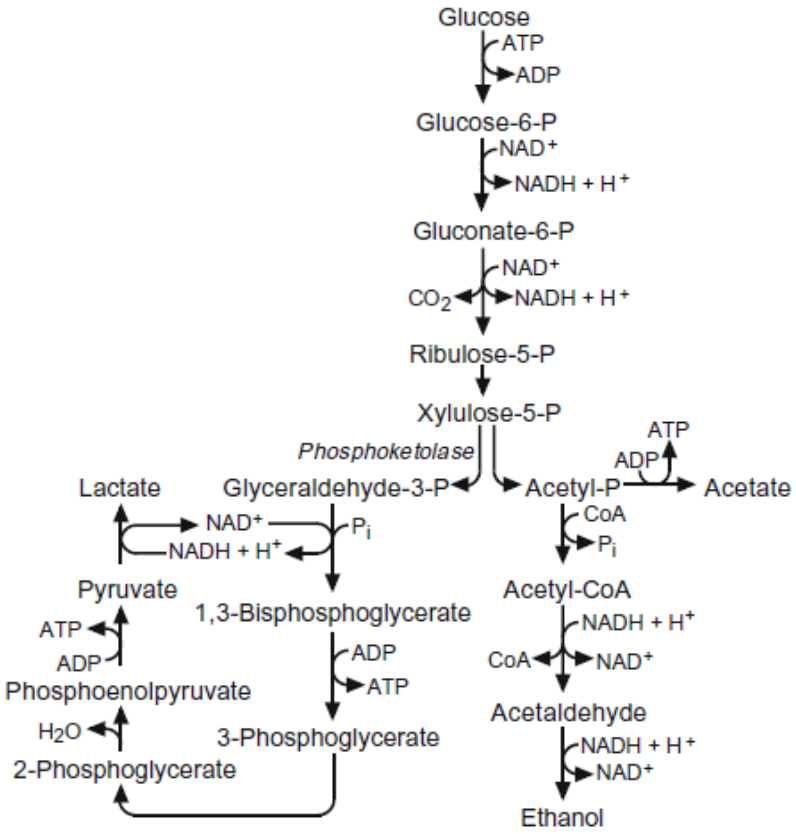


Figure 2.3 Heterofermentative metabolism pathways of lactic acid bacteria

As shown above, chemical synthesis requires petroleum feedstock and drastic conditions, while fermentation process utilizes renewable feedstock such as cellulose, starch, and molasses. Therefore, the latter is considered a green process which incurs low substrate costs. Moreover, bacterial fermentation yields high productivity, while fungal fermentation gives 100% optical purity of L-lactic acid. Nonetheless, fungal fermentation is a complex process due to the morphological change during operation, which in turn leads to difficulties in operation and design. Therefore, to date, bacterial fermentation remains the preferable option (Castillo Martinez et al., 2013; Datta and Henry, 2006; Ghaffar et al., 2014; Thongchul, 2013).

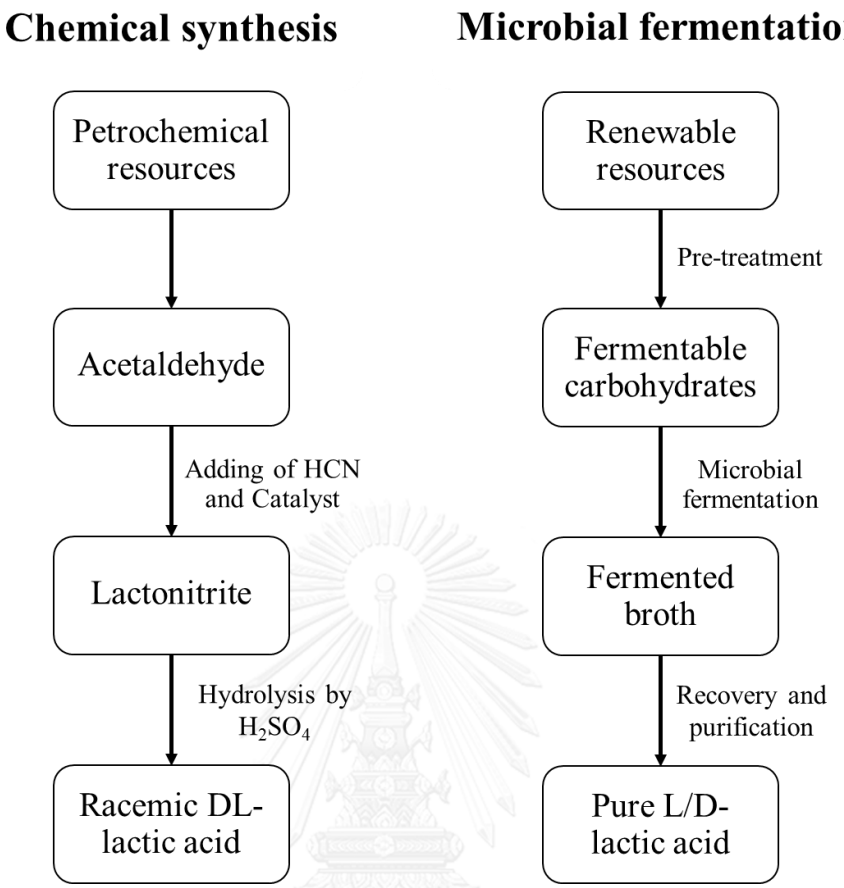


Figure 2.4 A schematic of chemical synthesis and microbial fermentation

There are many significant factors to lactic acid production by microbial fermentation such as strain of microbe, nutritional requirement (e.g. carbon source, nitrogen source, mineral, and amino acid), neutralizing agent, substrate, aeration, and fermentation processes.

2.4 Fermentation processes

There are three main processes of fermentation: batch fermentation, fed-batch fermentation, and continuous fermentation.

2.4.1. Batch fermentation

Industrially batch fermentation has been typically used for lactic acid production. The batch process is considered easy as there is no addition/removal of substrates and/or products during fermentation, except for neutralizing agents for pH control; thus, the process design is simple and there exists a low risk of contamination. However, the batch process usually suffers from substrate repression and end product inhibition. As a result, the high final product titer is limited and the investment and operating costs increase (a series of fermenters are required along with the larger downstream unit equipment). A summary of the many studies of lactic acid production via batch fermentation is provided in Table 2.2 (Calabia et al., 2011; Gao et al., 2012; Ge et al., 2011; Moon et al., 2012; Qin et al., 2010; Zhou et al., 2013).

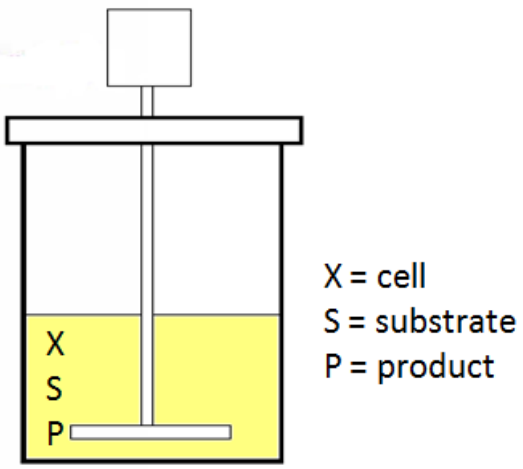


Figure 2.5 Batch fermentation process

Table 2.2 Lactic acid production in batch fermentation by different strains

Strain	Substrate	Lactic acid titer (g/L)	Yield (g/g)	Productivity (g/L·h)	Reference
Bacillus sp. Na-2	Glucose	106	0.94	3.53	Qin et al. (2010)
Rhizopus oryzae GY18	Glucose	115	0.81	1.6	Guo et al. 2010
	Xylose	68.5	0.85	0.57	
Rhizopus oryzae GY18	Sucrose	80.1	0.89	1.67	
Lactobacillus casei G-03	Glucose	198.2	0.94	5.5	Ge et al. (2011)
H. halophilus JCM 21694	Sucrose	65.8	0.83	1.1	Calabia et al. (2011)
Lb. paracasei subsp. paracasei CHB2121	Glucose	192	0.96	3.99	Moon et al. (2012)
Bacillus coagulans WCP10-4	Glucose	210.5	0.95	3.5	Zhou et al. (2013)

2.4.2 Fed-batch fermentation

The fed-batch process provides such benefits as low substrate repression and high cell and final product concentrations because in this approach the substrate is added during fermentation similar to what is done in the continuous process but without the broth draining out fermentation.

However, fed-batch fermentation still has some limitations concerning product inhibition resulting from high product accumulation.

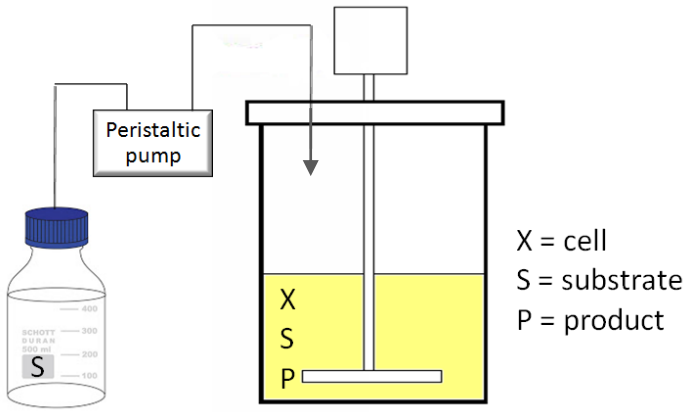


Figure 2.6 Fed-batch fermentation process

The fed-batch operation mode varies depending on the substrate feeding program. Different substrate feeding programs have been developed to serve the needs of various processes. These include the pulse fed-batch when the substrate is fed one at a time. A constant feed rate when the substrate is fed into the fermentation at a constant flow rate. A constant residual substrate concentration where during the operation the remaining substrate concentration remains constant. Finally, exponential feeding which is mostly used to maintain the cell yield that is able to find the feed rate according to the equation below.

$$F = \frac{\mu}{Y_{X/S}(S_i - S)} V_0 X_0 \exp(\mu t)$$

Where μ is the specific growth rate, $Y_{X/S}$ is the theoretical cell yield on the substrate, S_i and S are the substrate concentrations in the feeding solution and in the

reactor, V_0 is the initial volume, X_0 is the cell concentration and the initial cell concentrations, and t is the fermentation time.

In 2006, Ding and Tan studied lactic acid production by fed-batch fermentation using 4 different feeding programs compared with the batch process comprising pulse fed-batch fermentation, constant feed rate fed-batch fermentation, constant residual glucose concentration, and exponential fed-batch fermentation. The results showed that all 4 feeding programs gave higher lactic acid production compared to that obtained from the batch process (Ding and Tan, 2006). Li et al. (2010) compared lactic acid production from batch with that from the fed-batch operation. Via the fed-batch operation, the fermentation time was prolonged, resulting in a higher lactate concentration. However, the productivity when at the start of the fed-batch operation was slightly lower than that during the initial batch process (Li et al., 2010). As mentioned earlier, substrate repression is one of the key issues in batch operation. In 2012, Gao et al. attempted to decrease substrate repression in lactic acid fermentation by a pulse feeding program. A lower glucose concentration was intermittently fed into the fermentation during the operation. By this means productivity was improved and final lactate concentrations were obtained (Gao et al., 2012).

There are several other studies of batch fermentation compared with fed-batch fermentation, with some shown in Table 2.3. From Table 2.3 it can be seen that the most effective method is fed-batch fermentation. However, at the present, the productivity of the high L-lactic acid of 225 g/L obtained by multi-pulse fed-batch fermentation is still low according to Meng et al. (1.04 g/L·h) (2012) (Meng et al., 2012). So, in sum, a high final lactic acid concentration, yield, and productivity are required from fed-batch fermentation.

To achieve high product concentration in fed-batch fermentation, many factors should be considered such as the time of feeding the substrate, the substrate concentration to feed, the reminding substrate concentration, microorganism growth phase, and feeding method.

Table 2.3 Comparison of batch and fed-batch fermentation in L-lactic acid production

Fermentation study	Lactic acid titer (g/L)	% Yield	Productivity (g/L·h)	Reference
- Batch fermentation	112.5	88.6	1.34	Ding and Tan (2006)
- Pulse fed-batch	130.0	89.2	1.55	
- Constant feed rate fed-batch	135	87.6	1.61	
- Constant residual glucose fed-batch	152.5	92.8	1.82	
- Exponential fed-batch	157.5	91.2	1.88	
- Batch fermentation	90	69.23	-	Li et al. (2010)
- Fed-batch culture with glucose feedback control	152.5	-	-	
- Batch fermentation	143	90.3	2.75	Gao et al. (2012)
- Pulse fed-batch fermentation	183.2	98.5	3.52	
- Batch fermentation	19	95.0	-	Meng et al. (2012)
- Single-pulse fed-batch fermentation	154	98.6	1.61	
- Multi-pulse fed-batch fermentation	225	99.3	1.04	

Table 2.3 Comparison of batch and fed-batch fermentation in L-lactic acid production (continued)

Fermentation study	Lactic acid titer (g/L)	% Yield	Productivity (g/L·h)	Reference
- Batch fermentation	83.6	0.95	7.5	Ye et al. (2013)
- Multi-pulse fed-batch fermentation	215.7	99.6	4.00	

2.4.3. Continuous fermentation

The continuous process, on the other hand, helps minimize the substrate and product inhibition by gradually adding and removing the substrate and product at the same rate during the operation. Moreover, the continuous process is able to maintain the cells at a constant phase of growth and physiology, while maximum productivity can be obtained at a steady stage. Nonetheless, the benefits of operating the continuous culture has to balanced with the risks of contamination and mutation as the result of the long operation time as well as wash out due to the sudden change of process kinetics (Abdel-Rahman et al., 2013; Gao and Ho, 2013; Ohara et al., 1992).

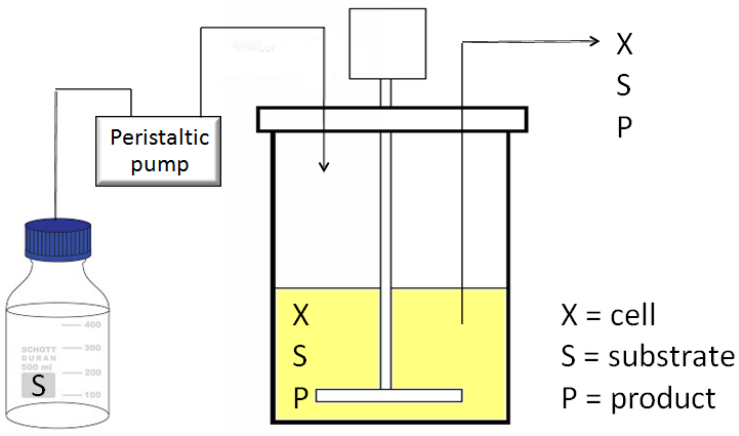


Figure 2.7 Continuous fermentation process

All of the fermentation strategies have advantages and some limitations as summarized in Table 2.4. Hence, the fermentation selection should consider the combinations of other factors, including microbial growth, the nature of the substrate, and the viscosity of the fermentation broth.

Table 2.4 Advantages and disadvantages of fermentation processes

Fermentation processes	**Advantages**	**Disadvantages**
Batch fermentation	- Easy operation - High product concentration - Reduced risk of contamination	- Low productivity - Substrate and/or end product inhibition
Fed-batch fermentation	- Overcome substrate inhibition problem - High product concentration - Labor-saving	- End product inhibition - Difficult to conduct optimal design
Continuous fermentation	- High productivity - Control growth rates - Less frequency shut down process	- Incomplete utilization of the carbon source

CHAPTER 3

EXPERIMENTAL

3.1 Apparatus and Chemicals

3.1.1 Apparatus

Apparatus	**Model**	**Manufacturer**	**Country**
Autoclave	KT-40L	ALP Co., Ltd.	Japan
Centrifuge	MC-15A	Tomy Seiko Co., Ltd.	Japan
Electronic balance	ML204/01	Metter Toledo AG	Switzerland
Electronic balance	ML3002E/01	Metter Toledo AG	Switzerland
High Performance Liquid Chromatography	Shimadzu LC-10A	Shimadzu Co., Ltd.	Japan
Laminar flow hood	NK system clean bench	International Scientific Supply	Thailand
Oven	UL-80	Memmert Co., Ltd.	Germany
pH meter	AB15	Fisher Scientific, Ltd.	Singapore
Rotary incubator shaker	G25	New Brunswick Scientific Co., Inc.	USA

Apparatus	**Model**	**Manufacturer**	**Country**
Vortex mixer	K-550-GE	Scientific Industries, Inc.	USA
Fermentor (5 Liter)	MDL 300	B.E. Marubishi	Thailand
Spectrophotometer	UV-1280	Shimadzu	Japan
Scanning Electron Microscope	JSM-6610 SEM	Evans Analytical Group	Germany

3.1.2 Chemicals

Chemicals	**Manufacturer**	**Country**
Agar	Patanasin Enterprise	Thailand
Ammonium chloride (NH_4Cl)	Riedel-de Haen	Germany
Ammonium sulfate $(NH_4)_2SO_4$	Merck	Germany
Calcium carbonate ($CaCO_3$)	Sigma	Germany
Calcium hydroxide ($Ca(OH)_2$)	Fluka	France
Copper sulfate ($CuSO_4 \cdot 5H_2O$)	Fluka	France
Ethanol (C_2H_5OH)	Merck	Germany
Glucose ($C_6H_{12}O_6$)	Siamchai Chemical	Thailand
Hydrochloric acid (HCl)	Merck	Germany
Iron sulfate heptahydrate ($FeSO_4 \cdot 7H_2O$)	Merk	Germany
Magnesium sulfate heptahydrate ($MgSO_4 \cdot 7H_2O$)	Riedel-de Haen	Germany

Chemicals	**Manufacturer**	**Country**
Peptone	Fluka	France
Potassium dihydrogen phosphate (KH_2PO_4)	Riedel-de Haen	Germany
Potassium phosphate dibasic (K_2HPO_4)	Riedel-de Haen	Germany
Sodium chloride (NaCl)	Sigma	Germany
Sodium hydroxide (NaOH)	Grand Chemical	Thailand
Yeast extract	Bio springer	France

3.2 Microorganism and inoculum preparation

3.2.1 Microorganism

Novel *Bacillus aerolacticus* BC-001 screened from the natural resources, a lactic acid producing bacterium, was used in this study. The culture was grown under aerobic condition at 50 °C for 1 day on GYP agar slant. To maintain the activity, the culture was transferred onto the freshly new GYP agar slant every week.

3.2.2 Inoculum preparation

1 mL sterile NaCl (0.85 %wt) was transferred into the fully grown culture onto GYP slant. The slant was thoroughly mixed to obtain the bacterial suspension. The optical density of the suspension was measured at the wavelength of 600 nm. The bacterial suspension was diluted with sterile NaCl (0.85 %wt) to the approximate OD600 of 30-40. 0.5 mL of the suspension was transferred into the 250 mL Erlenmeyer

flask containing 49.5 mL preculture medium. The culture was incubated at 50 °C, 200 rpm for 3 h.

3.3 Medium composition

3.3.1 GYP agar medium (per liter)

Glucose	10	g
Yeast extract	5	g
Peptone	5	g
KH_2PO_4	0.25	g
K_2HPO_4	0.25	g
$CaCO_3$	5	g
Agar	20	g
Salt solution	10	mL

3.3.2 Preculture medium for flask fermentation (per liter)

Glucose	10	g
Yeast extract	15	g
NH_4Cl	4	g
KH_2PO_4	0.5	g
K_2HPO_4	0.5	g
$CaCO_3$	5	g
Salt solution	20	mL

3.3.3 Preculture medium for 5 L stirred fermentor (per liter)

Glucose	10	g
Yeast extract	15	g
NH_4Cl	4	g
KH_2PO_4	0.5	g
K_2HPO_4	0.5	g
$Ca(OH)_2$	1	g
Salt solution	10	mL

3.3.4 Batch fermentation medium for flask fermentation

Initial glucose concentration (g/L)	**Glucose (g/L)**	**$CaCO_3$ (g/L)**
100	200	56
125	250	69.56
150	300	83.57
175	350	97
200	400	111.09

3.3.5 Batch fermentation medium for 5 L stirred fermentor

Initial glucose concentration (g/L)	**Glucose (g/L)**
100	400
125	500
150	600
175	700
200	800

3.3.6 Intermittent fed-batch solution for flask fermentation contained solely glucose powder at 25, 50, 75, and 100 g/L

3.3.7 Fed-batch fermentation solution for 5 L stirred fermentor

	C/N ratio	**Glucose (g/L)**	**NH_4Cl (g/L)**	**$(NH_4)_2SO_4$ (g/L)**
Glucose		500		
Glucose +	20	500	38.19	
NH_4Cl				
Glucose +	20	500		47.17
$(NH_4)_2SO_4$	40	500		23.58
	60	500		15.71
	80	500		11.79

3.3.8 Salts solution (in 10 mL)

$MgSO_4 \cdot 7H_2O$	400	mg
$MnSO_4 \cdot 5H_2O$	20	mg
$FeSO_4 \cdot 5H_2O$	20	mg
NaCl	20	mg

All media were autoclaved at 121 °C, 15 psig for 15 min.

3.4 Methodology

3.4.1 L-lactic acid production in flask fermentation

3.4.1.1 Determine the optimized initial glucose concentration in flask fermentation

50 mL concentrated glucose solution at different concentrations (200-400 g/L) was transferred into 50 mL preculture inoculum prepared in 3.2.2 to bring up the concentration to 100-200 g/L. Equilibrate $CaCO_3$ was added into the fermentation culture for pH control. The culture was further incubated at 50 °C, 150 rpm. Samples were taken every 3 h until lactic acid concentration remained constant or decreased.

3.4.1.2 Determine the optimized feeding glucose concentration via Intermittent fed-batch fermentation

50 mL concentrated glucose solution at 200 g/L was transferred into 50 mL preculture inoculum prepared in 3.2.2 to initiate batch fermentation. 5.6 g $CaCO_3$ was

added into the fermentation culture for pH control. Fed-batch operation started when the glucose concentration fell in between 50 and 60 g/L; glucose powder at 25, 50, 75, and 100 g/L was added. The culture was further incubated at 50 °C, 150 rpm. Samples were taken every 3 h until lactic acid concentration remained constant or decreased.

3.4.1.3 Intermittent feeding fed-batch fermentation

Concentrated glucose solution (200 g/L, 50 mL) was transferred into 50 mL preculture inoculum obtained from 3.2.2 to initiate batch fermentation. $CaCO_3$ powder (5.6 g) was added into the fermentation culture for pH control. The culture was incubated at 50 °C, 150 rpm. Samples were taken every 3 h to check for the remaining glucose. Fed-batch operation started when the glucose concentration fell in between 50 and 60 g/L. 2.5 g glucose powder and 5.6 g $CaCO_3$ were added into the fermentation culture repeatedly. The fermentation was carried out for 48 h.

3.4.2 Determine the optimized initial glucose concentration in a 5 L stirred fermentor (batch fermentation)

Lactic acid fermentation was conducted in a 5 L stirred fermentor (B.E. Marubishi (Thailand) Co., Ltd.). Before autoclave, the pH probe was calibrated with the technical buffers (pH 4 and pH 7). The fermentor was filled up with 1.35 L preculture medium (with initial glucose concentration of 15 g/L after inoculation). The fermentor was sterilized in an autoclave at 121 °C, 15 psig for 30 min.

After autoclave, the fermentor was allowed to cool down to 50 °C. Later, DO calibration was conducted at this operating temperature. The fermentor was agitated at

300 rpm with 0.1 vvm air supply. The inoculum prepared in 3.2.2 (0.15 L) was aseptically transferred into the fermentor. The preculture in fermentor was carried out for 3 hs. To initiate batch fermentation, the aeration was stopped and 0.5 L of the fermentation medium: glucose solution at different concentrations of 400-800 g/L, was transferred into the fermentor to bring up the concentration to 100-200 g/L. During fermentation, the fermentor was operated at the same temperature and agitation. The pH was automatically controlled at 6.5 by 10 M NaOH. The fermentation was preceded until glucose depletion or lactic acid production stopped. Samples were taken every 3 h for analyses of cell biomass, remaining glucose, lactate, and byproducts.

3.4.3 Conduct fed-batch fermentation using different feeding programs in the 5 L stirred fermentor

All fed-batch cultures were started from batch fermentations (see 3.4.2) at the initial glucose concentration of 100 g/L.

3.4.3.1 Intermittent fed-batch fermentation

Using the kinetic data from batch culture, intermittent fed-batch operation was started after batch culture was conducted until approaching high lactate production rate. 50 mL of concentrated glucose solution (500 g/L) was intermittent fed into the fermentation culture every 3 h until lactate production stopped or the accumulation in the fermentor reached 85-90% tank volume. Fermentor was operated at the same conditions as those in 3.4.2. During this fed-batch operation, samples were taken every 3 h for analyses.

3.4.3.2 Constant mass flow rate fed-batch fermentation

Constant mass flow rate fed-batch fermentation was started after batch cultivation was conducted in the similar manner to that for intermittent feeding program. Concentrated glucose solution (500 g/L) was constantly fed into the fermentor at 10 g/L·h (precalculated using the kinetics data obtained from intermittent fed-batch operation in 3.4.3.1). Fermentation was continued until lactate production stopped or the accumulation in the fermentor reached 85-90% tank volume. During this fed-batch operation, samples were taken every 3 h for analyses.

3.4.3.3 Constant feed rate fed-batch fermentation

Similarly to constant mass flow rate fed-batch fermentation, constant feed rate fed-batch fermentation was started after batch cultivation was conducted in the similar manner to that for intermittent feeding program. Concentrated glucose solution (500 g/L) was constantly fed into the fermentor at 10 g/L·h (precalculated using the kinetics data obtained from intermittent fed-batch operation in 3.4.3.1). Fermentation was continued until lactate production stopped or the accumulation in the fermentor reached 85-90% tank volume. During this fed-batch operation, samples were taken every 3 h for analyses.

The assumption used in determining the feed rate for was set as seen below:

Total glucose consumption rate = Rate of glucose consumed for cell (μ and $Y_{x/s}$) + Rate of glucose consumed for lactate (lactate productivity, $Y_{p/s}$, and $Y_{p/x}$)

3.4.4 Determine the optimized nitrogen source and C/N ratio in fed-batch fermentations

Using the intermittent, constant mass flow rate, and constant feed rate programs for fed-batch cultivation previously described in 3.4.3.1, the feeding solution was varied from sole glucose solution to glucose supplemented with different nitrogen sources (NH_4Cl and $(NH_4)_2SO_4$ at different C/N ratios. Fed-batch fermentation was conducted in the similar manner to those mentioned in 3.4.3.1. During operation, samples were taken every 3 h for analyses.

3.4.5 Sample analyses

3.4.5.1 Optical density (OD)

Cell biomass was determined by mean of optical density at 600 nm. In flask fermentation study, for preculture broth, the sample was mixed with 1 M HCl at a ratio of 1 : 1 while for the fermentation broth, the sample was mixed with 1 M HCl at a ratio of 1 : 9 (to total volume of 1 mL). The mixture was thoroughly mixed to ensure solubilization of the remaining calcium bases. For the sample broth obtained from the studies in the 5 L stirred fermentor, the sample was mixed with 1 M HCl at the ratio of 1:1 to dissolve the remaining $Ca(OH)_2$. After acidification by HCl, the sample was centrifuged at 10000 g for 5 min. The supernatant was collected for analyses of the remaining glucose, lactate, and byproducts while the cell pellet was resuspended in 1 mL DI water. The pellet suspension was thoroughly vortexed and the optical density was measured at the wavelength of 600 nm using a spectrophotometer.

3.4.5.2 Analytical methods

3.4.5.2.1 High performance liquid chromatography (HPLC)

High performance liquid chromatography was used to analyze the fermentation broth sample for the remaining glucose, lactate, and byproducts. Before analyzing, the broth sample was filtered through hydrophilic PTFE membrane (0.45 μm pore size) and diluted with double distilled water. Diluted particle-free sample (15 μL) was injected into an organic acid analysis column (Biorad, Aminex HPX-87H ion exclusion organic acid column; 300 mm × 7.8 mm) maintained at 45 °C in a column oven. 0.005 M H_2SO_4 was used as an eluant at the flowrate of 0.6 mL/min. A refractive index detector was used to detect the organic compounds. Standards containing glucose, lactate, and byproducts including ethanol and acetate at different concentrations in between 0 and 2 g/L were injected as the references. The peak area was used for the comparison basis.

3.4.5.2.2 Glucose-lactate analyzer

Remaining glucose in the solution samples collected from fed-batch fermentation was analyzed by glucose-lactate analyzer (YSI 2700) before adding glucose solution. The particle-free sample was diluted with DI water before reading by the analyzer.

3.4.5.3 Scanning electron microscope (SEM)

Cell morphology of *B. aerolacticus* BC-001 during fermentation and lactate recovery was analyzed and compared with that in batch fermentation. Fermentation broth was filtered through 0.2 μm membrane (Merck Millipore membrane) to collect the biomass of *B. aerolacticus* BC-001. The sample on the membrane was then fixed by immersing in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 6.8-7.4) overnight. The sample was thoroughly rinsed with 0.1 M phosphate buffer (pH 7.2) for 5-10 min followed by DI water for another 5-10 min and progressively dehydrated in various concentrations of ethanol (from 30%, 50%, 70%, 80%, 90%, to 100%) for 5-10 min at each concentration. To prevent the damage of the specimen, a critical point dryer (CPD 020, Balzer) was used to dry to specimen to the critical drying point. Before examination, the sample was coated with electrically conductive material (60% gold and 40% palladium) using a spotter coating machine (CPD 020, Balzer) in the presence of the medium containing argon gas. After coating, the sample was examined using the scanning electron microscope (JSM-5410LV, JEOL).

CHAPTER 4

RESULTS AND DISCUSSION

4.1 L-lactic acid production in flask fermentation

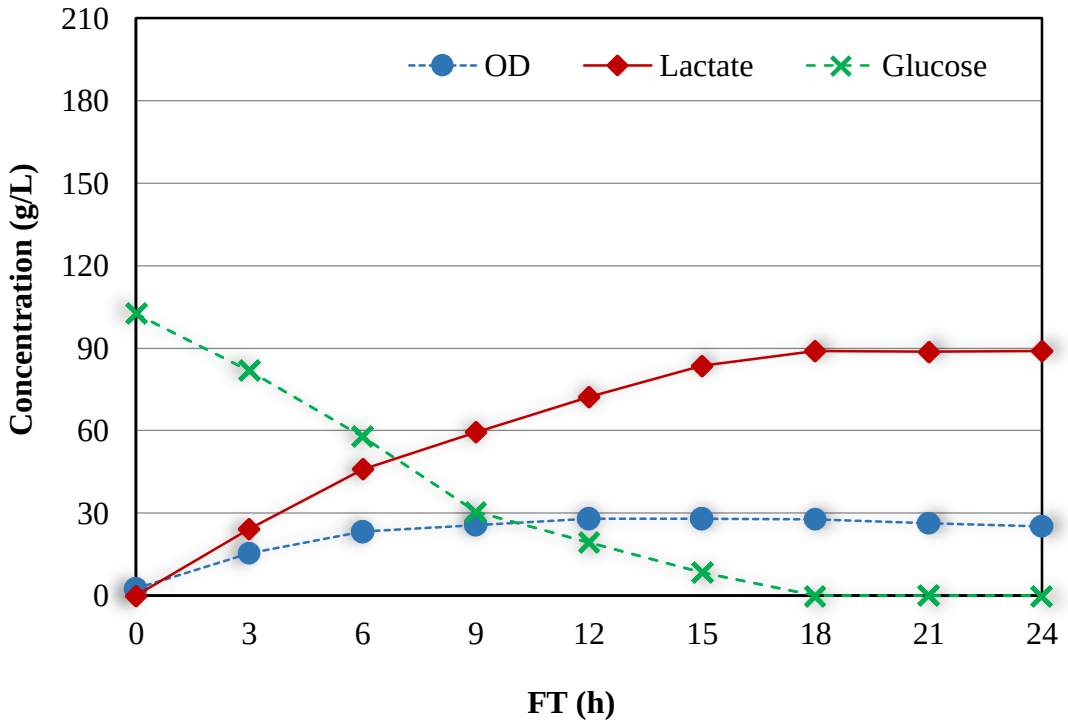
4.1.1 Effect of different initial glucose concentration on L-lactic acid production via batch fermentation in shake flask by *B. aerolacticus* BC-001

The simple way to achieve high final lactate concentration at the end of fermentation is to ferment at high initial substrate concentration. In order to establish the industrial fermentation platform, the simple operation is mandatory. Therefore, *B. aerolacticus* BC-001 was tested for batch fermentation in shake flask using initial glucose at high concentration under the condition of mixing at 150 rpm and 50 °C. At high temperature such that the risk of microbial contamination could be reduced due to most of microorganisms prefer to grow at 25-45 °C (mesophilic) (Abdel-Banat et al., 2010). Figure 4.1 shows the fermentation profiles of lactate fermentation by *B. aerolacticus* BC-001 at different initial glucose concentrations. From the fermentation profiles, no acetate formation was observed although mixing at 150 rpm was introduced during fermentation period. This somewhat showed that unlike other *Bacillus* spp. that often experienced acetate formation when mixing was applied during fermentation. It has been reported that *B. coagulans* C106 produced pure L-lactic acid with the optimal temperature at 50 °C under strictly anaerobic condition. Ye et al. remarked that it might be owing to the frameshift mutation of D-lactate dehydrogenase gene rendering to high optical purity of L-lactic acid (Ye et al., 2013). *B. aerolacticus* BC-001 was capable of

fermenting lactate under slightly aerobic condition. It should be noted that mixing at 150 rpm provided beneficial outcome in term of homogeneity of the broth which contained insoluble $CaCO_3$ thus pH control was improved. At the beginning of fermentation cell biomass increased rapidly until 12 h. After that the OD reading was slightly changed. From Figure 4.1, it can be seen that *B. aerolacticus* BC-001 was able to ferment glucose at all concentrations studied for lactic acid but at slightly different consumption rate. It was found that at initial glucose concentrations of 100-175 g/L, glucose was completely consumed while at 200 g/L initial glucose, some glucose was remained at the end of fermentation. Increasing initial glucose concentration up to 175 g/L resulted in higher final lactate. Compared with other strains, there are a few of thermophilic lactic acid bacteria that have been reported to be able to tolerate high substrate concentration as high as 130 g/L glucose or xylose. Those include, for instance, *Latobacillus paracasei* subsp. *paracasei* CHB212 (Moon et al., 2012), *Latobacillus casei* mutant G-03 (Ge et al., 2011), and *Bacillus* sp. WL-S20 (Meng et al., 2012). Further increasing initial glucose concentration to 200 g/L on the other hand gave lower final lactate titer. This is somewhat indicated glucose repression in fermentation by *B. aerolacticus* BC-001 at the concentration higher than 175 g/L. Another evidence supporting glucose repression at the initial concentration was observed from the lag phase of lactate formation for 3 h (Figure 4.1 (E)) while no lag phase appeared in other concentrations studied (Liu et al., 2005; Taleghani et al., 2014; Zhou et al., 2013). By the way, Zhou et al. reported *Bacillus coagulans* WCP10-4, newly isolated, was able to ferment 240 g/L initial glucose to 210 g/L L-lactic acid with the yield and productivity of 95% and 3.5 g/L·h, respectively (Zhou et al., 2013).

Table 4.1 show the fermentation kinetics of *B. aerolacticus* BC-001. It was found that at higher initial concentration, longer time was required until glucose depletion. Not only higher lactate titer was obtained at higher initial glucose concentration, the yield was also increased. While the productivity was gradually dropped. Lowering lactate productivity was also the evidence indicating the chance of glucose repression when higher initial glucose concentration was used in fermentation (Zhou et al., 2013). The highest final lactate titer with the corresponding high yield and productivity was obtained at the initial glucose concentration of 175 g/L.

(A)


OD
Lactate
Glucose
Concentration (g/L)
FT (h)
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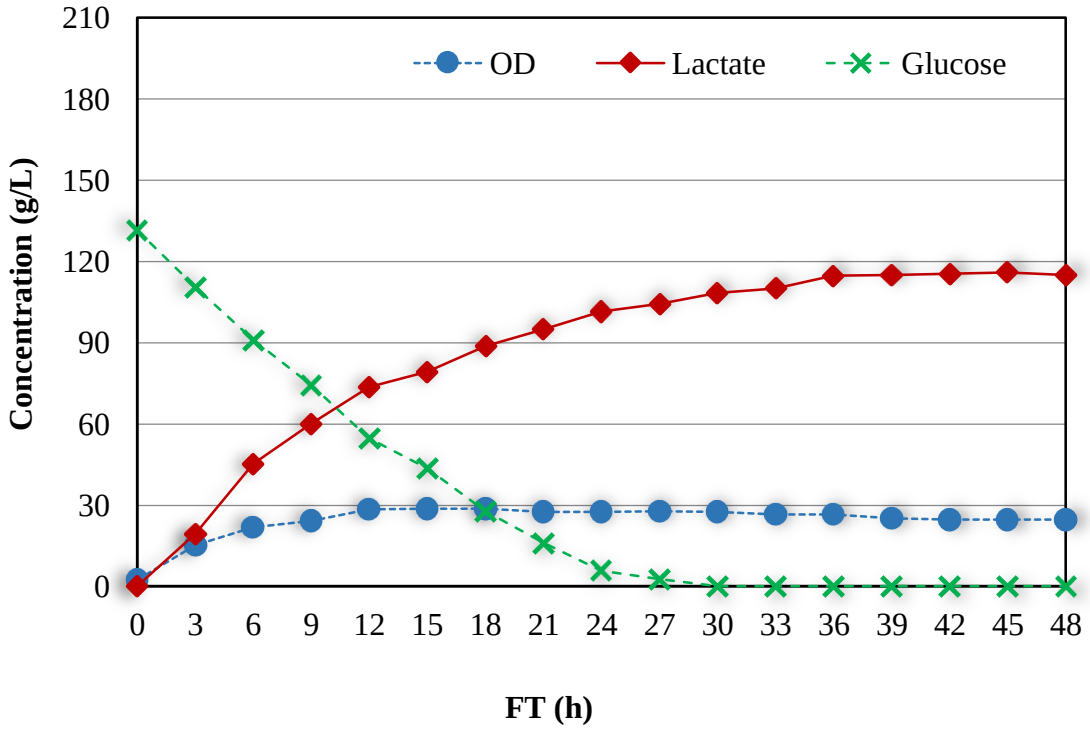
30
60
90
120
150
180
210
0
3
6
9
12
15
18
21
24

(B)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Lactate
Glucose
Concentration (g/L)
FT (h)
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30
60
90
120
150
180
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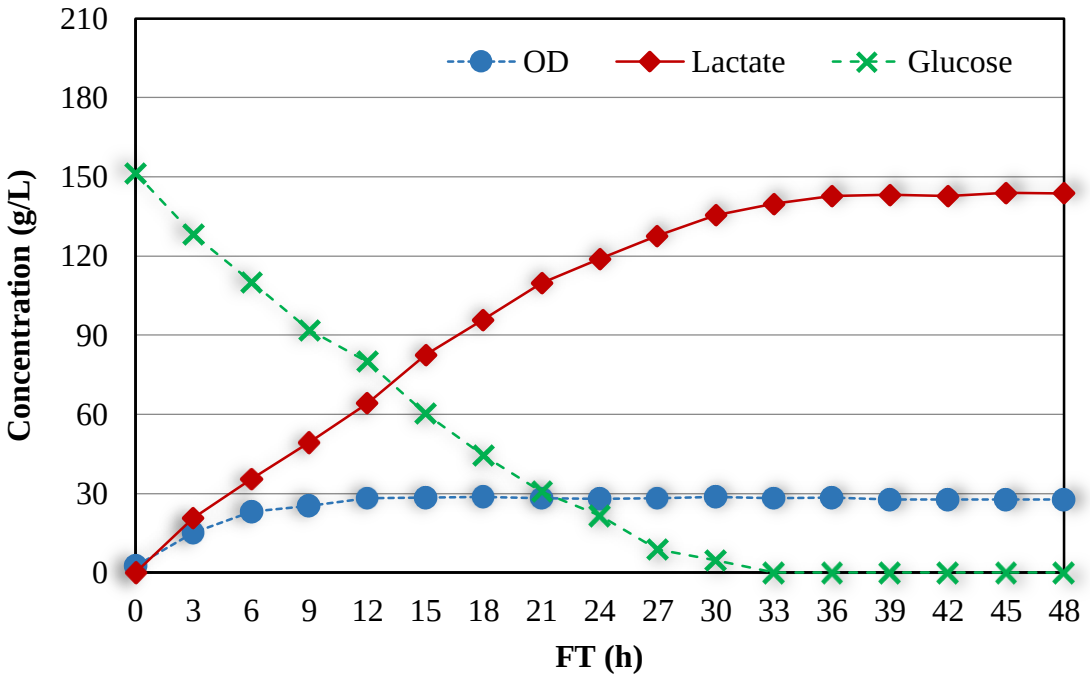
30
33
36
39
42
45
48

(C)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Lactate
Glucose
Concentration (g/L)
FT (h)
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30
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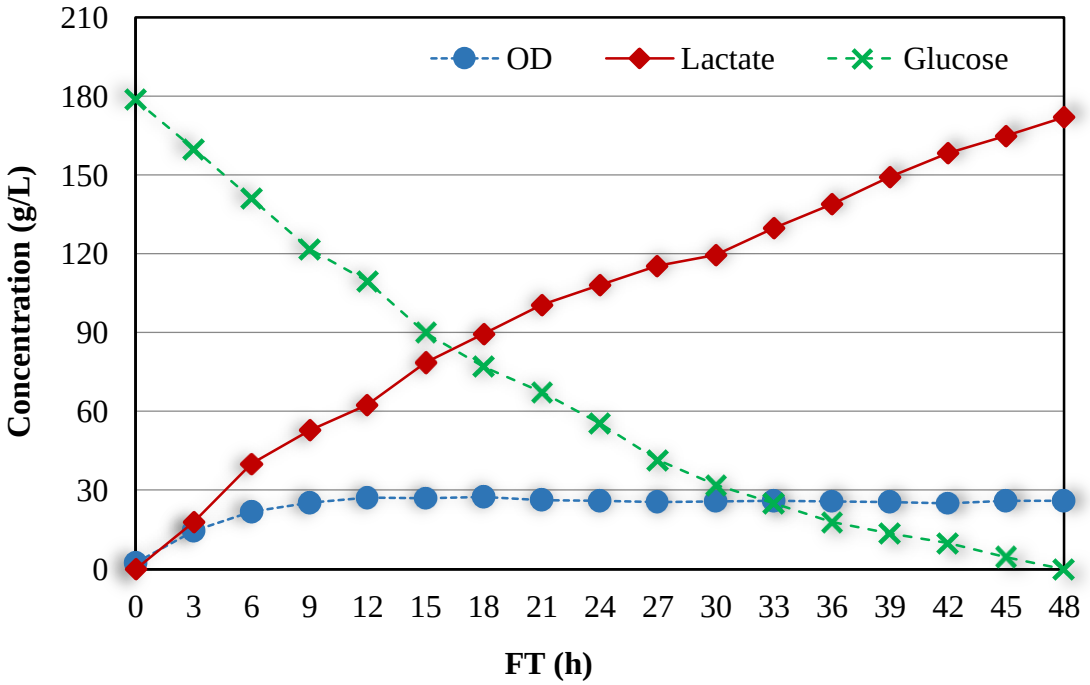
90
120
150
180
210
0 3 6 9 12 15 18 21 24 27 30 33 36 39 42 45 48

(D)


OD
Lactate
Glucose
Concentration (g/L)
FT (h)
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90
120
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0 3 6 9 12 15 18 21 24 27 30 33 36 39 42 45 48

(E)

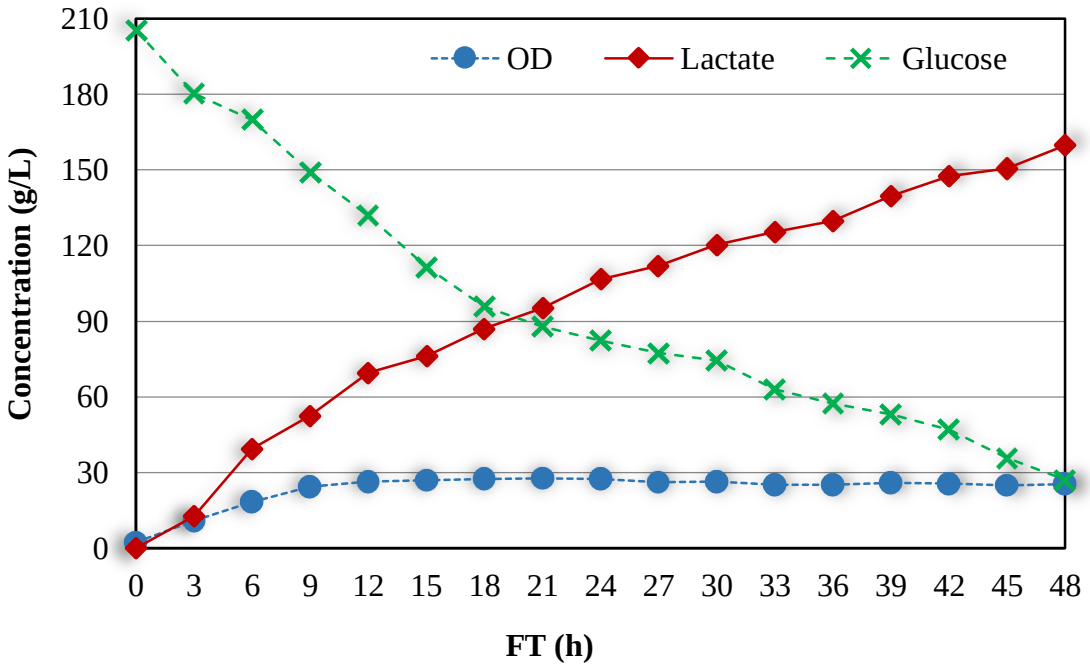


Figure 4.1 Fermentation profiles of *B. aerolacticus* BC-001 grown in glucose based medium at different glucose concentrations; 100 g/L (A), 125 g/L (B); 150 g/L (C); 175 g/L (D); and 200 g/L (E) in shake flask.

Table 4.1 Effect of initial glucose concentration on L-lactic acid production by *B. aerolacticus* BC-001

Initial glucose (g/L)	When glucose depleted					When achieving highest lactate titer				
	Time (h)	L-lactate titer (g/L)	Yield (g/g)	Productivity (g/L/h)	OD_{600}	Time (h)	Lactate titer (g/L)	Yield (g/g)	Productivity (g/L/h)	OD_{600}
100	18	89.05	0.89	4.95	27.70	18	89.05	0.89	4.95	27.70
125	30	108.52	0.87	3.62	27.53	36	114.89	0.92	3.19	26.53
150	33	139.91	0.93	4.24	28.10	39	143.33	0.96	3.96	27.80
175	48	172.07	0.98	3.58	26.00	48	172.07	0.98	3.58	26.00
200						48	159.65	0.92	3.33	25.47

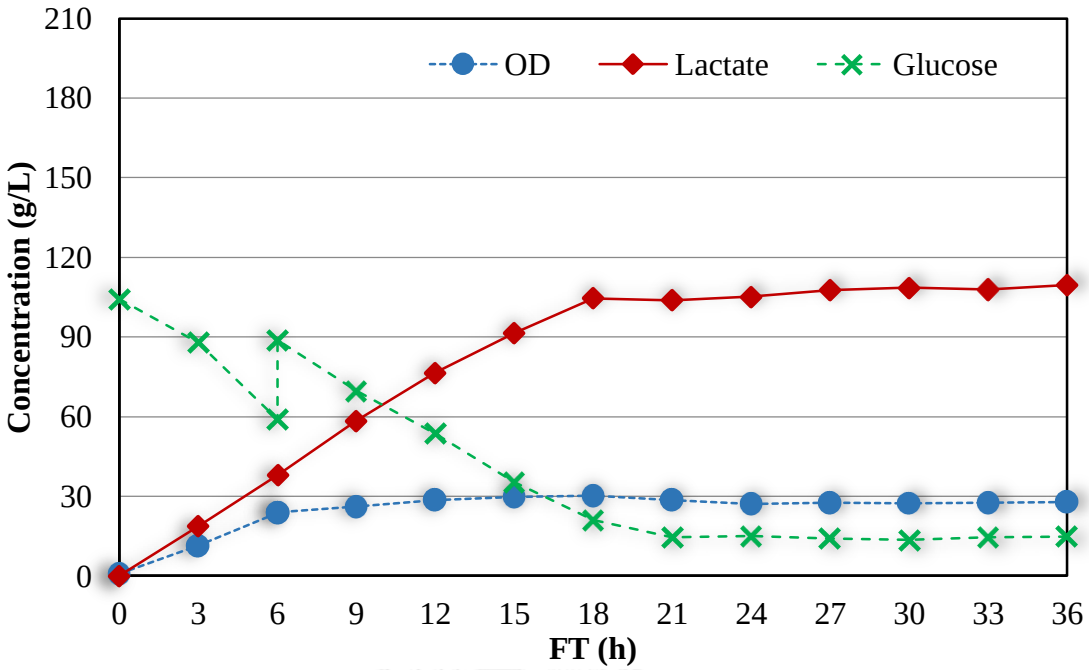
4.1.2 Effect of different feeding glucose concentration via intermittent fed-batch fermentation on L-lactic acid production in shake flask by *B. aerolacticus* BC-001

In order to limit glucose repression while obtaining high final lactate titer with the sufficiently high yield and productivity as observed during batch operation at low initial glucose concentration, fed-batch operation was introduced in fermentation by *B. aerolacticus* BC-001. As a result from table 4.1, when achieving highest lactate titer, the yields were not different. By the way productivity of lactic acid production from 100 g/L initial glucose concentration was highest, therefore 100 g/L initial glucose was selected to the next step in determining the proper glucose concentration to feed at the time that high productivity achieved. Glucose solution at different concentration (25, 50, 75, and 100 g/L) was fed after 6 h.

According to Figure 4.2, glucose uptake rate, lactic acid production rate, and biomass were dropped when adding glucose powder at the concentration higher than 25 g/L. Adding 100 g/L glucose powder led to lag phase in the beginning of fed-batch fermentation obviously (Figure 4.2 (D)). Lactic acid production was decelerate when lactic acid achieve approximately 100 g/L as $CaCO_3$ was added equilibrate to 100 g/L lactic acid.

Table 4.2 show that at the initial batch fermentation (0 to 6 h) production rate were approximately 6-7 g/L·h. Adding 25 g/L glucose powder was able to maintain production rate at 5 g/L·h during fed-batch operation. This value was close to that obtained from initial batch fermentation. Moreover, adding 25 g/L was also increase production rate during 6-18 h from normal batch fermentation with 100 and 125 g/L initial glucose concentration; 3.59 and 3.63 g/L·h, respectively).

(A)


OD
Lactate
Glucose
Concentration (g/L)
FT (h)
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30
60
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210
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12
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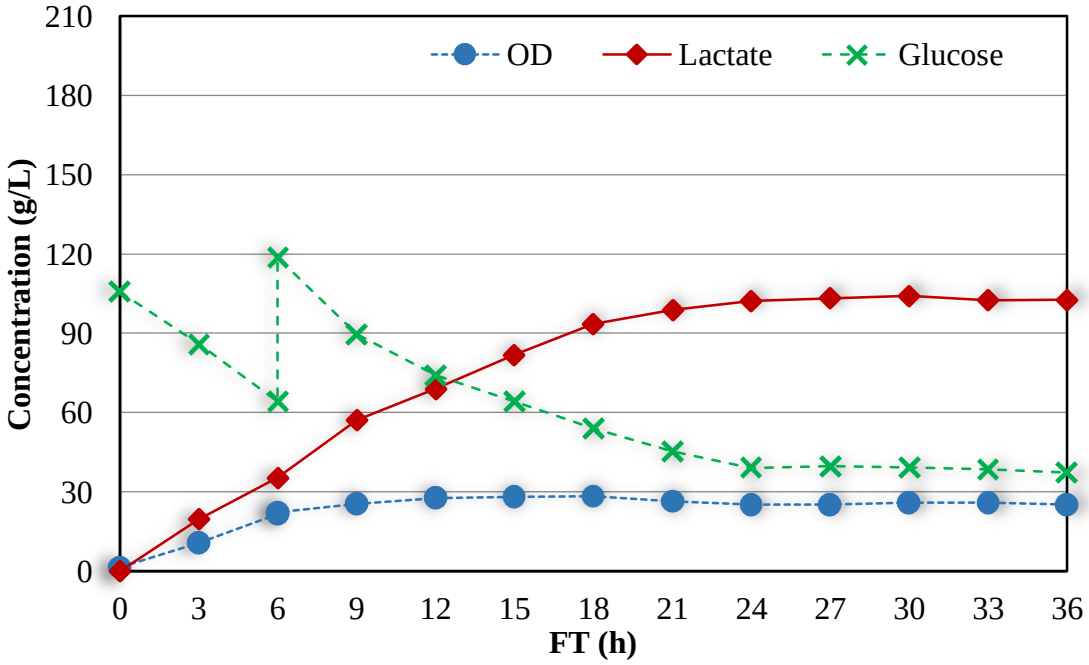
33
36

(B)


OD
Lactate
Glucose
Concentration (g/L)
FT (h)
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30
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0
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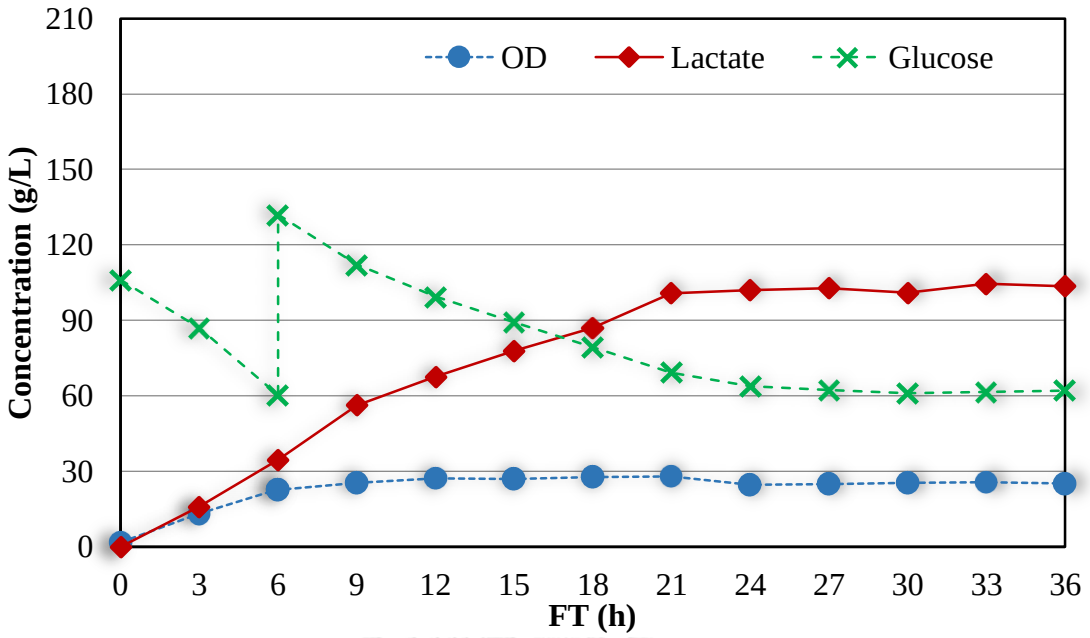
(C)



(D)

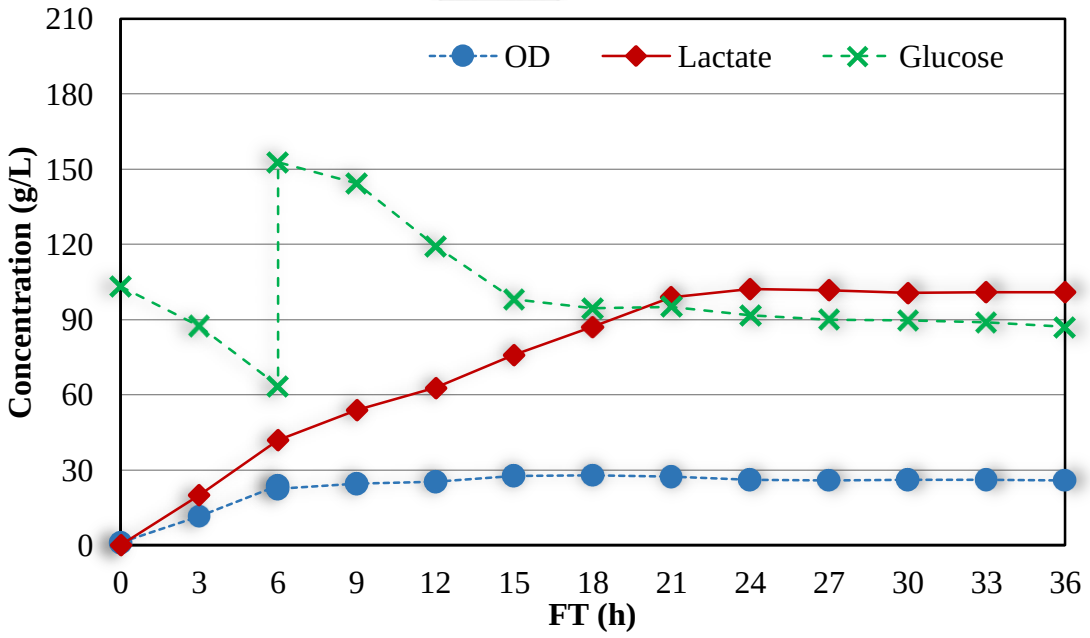


Figure 4.2 Fermentation profiles of *B. aerolacticus* BC-001 grown in glucose based medium at 100 g/L initial glucose concentrations while different feeding glucose concentration; 25 g/L (A), 50 g/L (B); 75 g/L (C); and 100 g/L (D) in shake flask.

Table 4.2 Comparison of batch and fed-batch in different total glucose concentration affected on L-lactic acid production

Initial glucose (g/L)	**Final lactate (g/L)**	**Productivity (g/L·h)**			**Yield (g/g)**	**Max OD_{600}**
		When achieving highest lactate	**0-6 h**	**6-18 h**		
100	89.05	4.95	7.66	3.59	0.89	27.97
125	114.89	3.19	7.53	3.63	0.92	28.80
150	143.33	3.96	5.92	5.03	0.96	28.67
175	172.07	3.58	6.70	4.12	0.98	27.50
200	159.65	3.33	6.55	3.99	0.92	27.83
100 + 25	92.45	5.81	6.87	5.28	0.92	30.20
100 + 50	102.19	4.26	6.31	4.64	0.84	28.43
100 + 75	102.06	4.25	6.12	4.19	0.90	27.83
100 + 100	102.11	4.25	6.52	4.00	1.01	27.83

4.1.3 Intermittent feeding fed-batch fermentation in shake flask by *B. aerolacticus* BC-001

Simple feeding program by intermittent feeding of glucose was applied. As shown in Figure 4.3, high final lactate titer of 192 g/L was acquired from fed-batch culture with the operating time of 54 h with the acceptable yield of 0.90 g/g and

productivity of 3.55 g/L·h which were comparable to those obtained from batch culture (Table 1). From the kinetics data obtained in fed-batch operation, this indicates that high final lactate titer could be obtained by the simple fed-batch operation. The result indicated that fed-batch fermentation was capable of reducing substrate inhibition occurred in batch fermentation. Nonetheless, lower glucose consumption rate occurred after the long operating time revealed that further optimization of the feeding program is required in order to improve better fermentation process efficiency (Ou et al., 2011). In fact, *B. aerolacticus* BC-001 could be able to produce more lactic acid; however, the fermentation had to be finished due to a large amount of calcium lactate salts (concentration higher than its solubility of 128 g/L at 50° C) leading to precipitate and turning the broth rheology to clay-like broth. As a result, it was difficult to obtain homogeneous mixing and sampling could not be done. but we have to discontinue the experiment because broth was like a clay that affect to defectively mixing and hard to sampling (Cao et al., 2001). In addition, there are also other disadvantages of using $CaCO_3$ include high sulfuric consumption from process of calcium lactate acidified to convert to lactic acid and calcium sulfate ($CaSO_4$), a waste byproduct that was adversary economy and ecology in waste treatment (Corma et al., 2007; Vaidya et al., 2005).

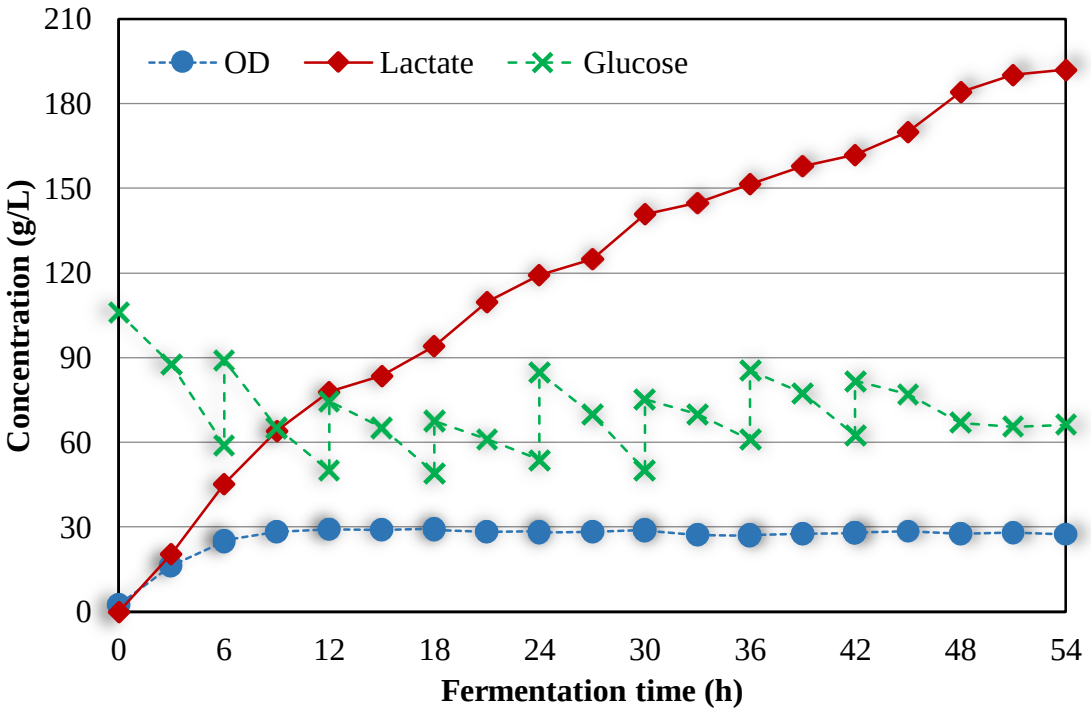


Figure 4.3 High final lactate titer obtained from fed-batch fermentation by *B. aerolacticus* BC-001 using intermittent feeding program of glucose.

4.2 Effect of different initial glucose concentration on L-lactic acid production via batch fermentation by *B. aerolacticus* BC-001 in 5 L stirred fermentor

In industry, fermentation platform is desired to meet high product concentration as that obtained in the laboratory scale test and easy operation (Schmidt, 2005). Therefore, the preliminary step in the small scale fermentor is mandatory to set up the platform. In these small scale runs, the optimal operating conditions and the protocol were set up.

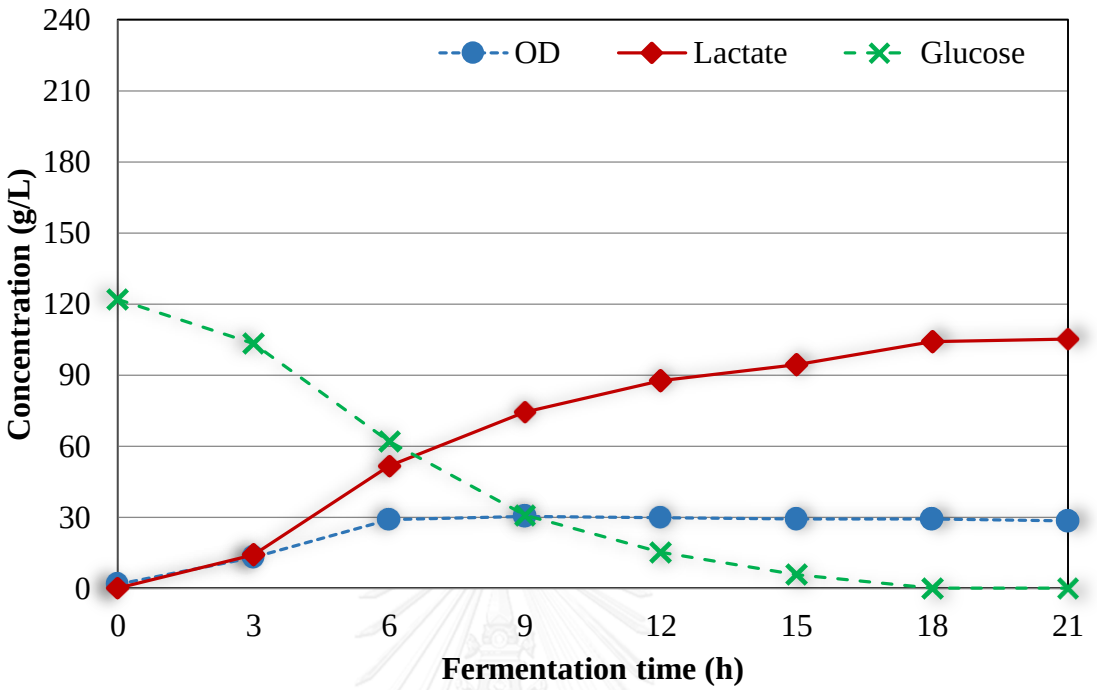
From the previous study conducted by Rampai (2015), fermentation optimization has been conducted in the batch mode in the 5 L stirred fermentor. The fermentor was inoculated with the 3 h preculture broth cultivated in the shake flask.

The fermentor was agitated at 300 rpm with 0.1 vvm air supply for another 3 h preculture period to increase inoculum concentration. Then the aeration was stopped and the pH was automatically controlled at 6.5 by 10 M NaOH to initiate batch lactate fermentation (Rampai, 2015).

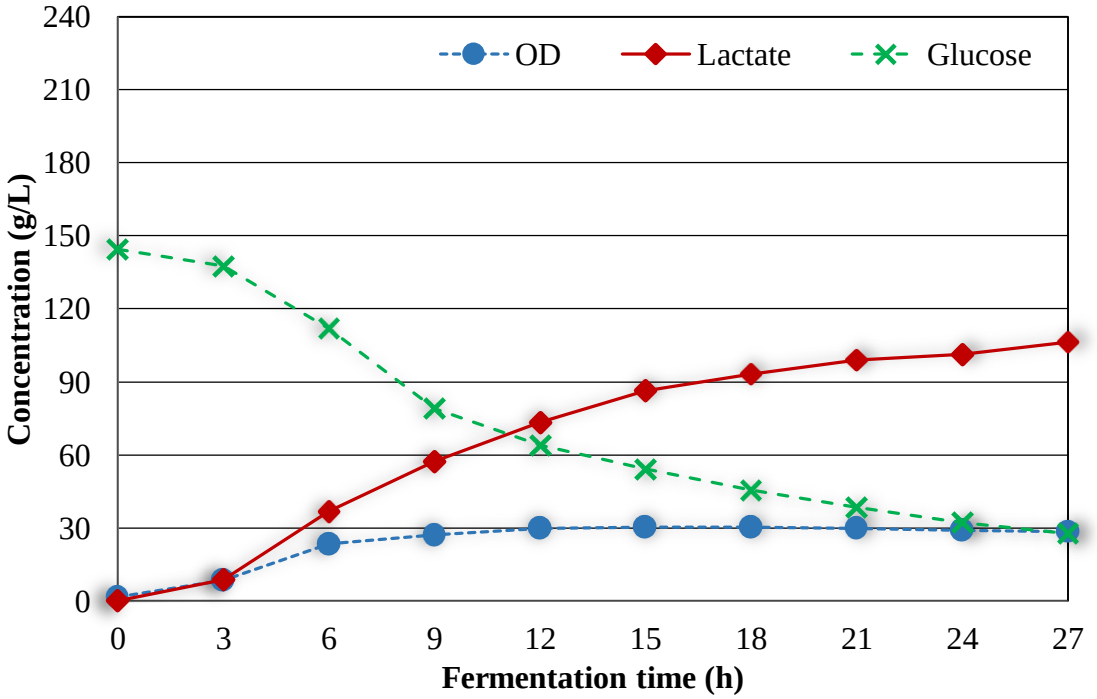
From above mentioned in 4.1.1, the simple way to achieve high final lactate concentration at the end of fermentation is to ferment at high initial substrate concentration. Batch fermentations in the 5 L stirred fermentor were also conducted at different initial glucose concentration from 100 g/L to 200 g/L (Figure 4.4). Glucose was completely consumed only in the fermentation using initial glucose concentration of 100 g/L while the lag phase was observed in other runs using higher initial glucose concentration.

The kinetics data in Table 4.3 confirmed that using initial glucose concentration higher than 100 g/L in the stirred fermentation lowered glucose uptake rate and lactate productivity. The remaining glucose appeared as the evidence of substrate repression caused by high osmotic pressure at high glucose concentration. It is noted that the water activity was reduced at this point with the corresponding plasmolysis (Ding and Tan, 2006; Gao et al., 2012; Kotzamanidis et al., 2002). Moreover, during batch fermentation study by Ye et al. (2013), lactate dehydrogenase activity was measured. They found that the enzyme activity was lower with initial high substrate concentration. This indicated the chance of substrate inhibition (Ye et al., 2013). Hereby fed-batch fermentation was suggested to overcome the initial substrate inhibition (Lee et al., 2007).

(A)


OD
Lactate
Glucose
Concentration (g/L)
240
210
180
150
120
90
60
30
0
0
3
6
9
12
15
18
21
Fermentation time (h)

(B)


OD
Lactate
Glucose
Concentration (g/L)
240
210
180
150
120
90
60
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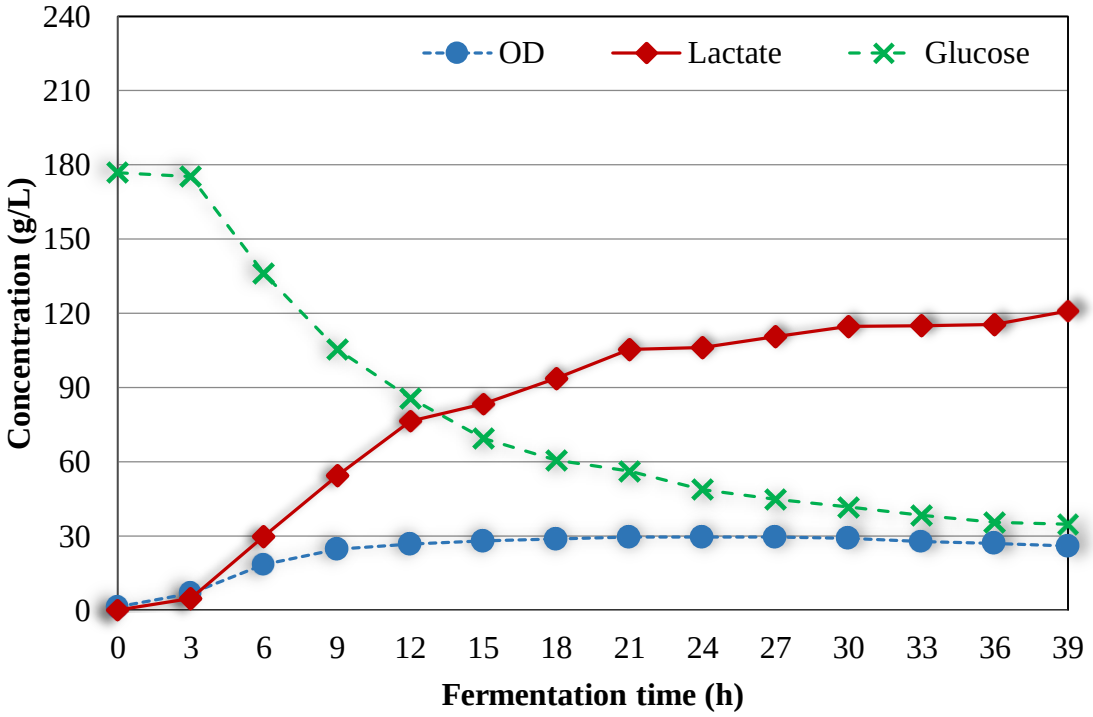
3
6
9
12
15
18
21
24
27
Fermentation time (h)

(C)


OD
Lactate
Glucose
Concentration (g/L)
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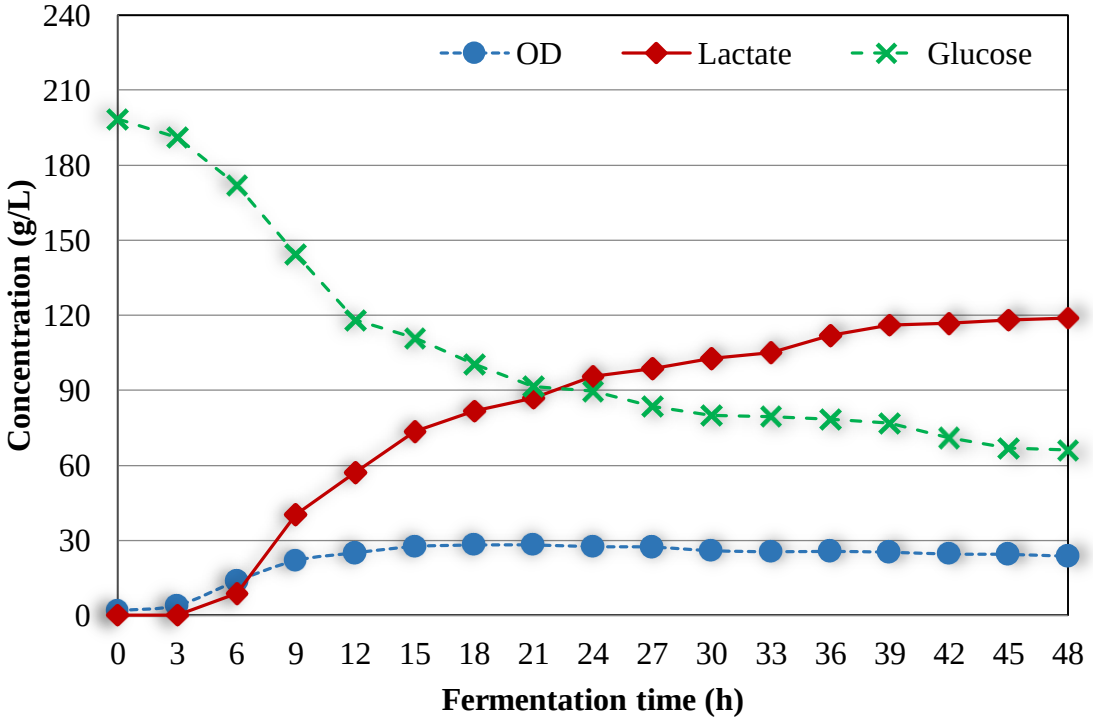
180
150
120
90
60
30
0
0 3 6 9 12 15 18 21 24 27 30 33 36 39
Fermentation time (h)

(D)


OD
Lactate
Glucose
Concentration (g/L)
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120
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60
30
0
0 3 6 9 12 15 18 21 24 27 30 33 36 39 42 45 48
Fermentation time (h)

(E)

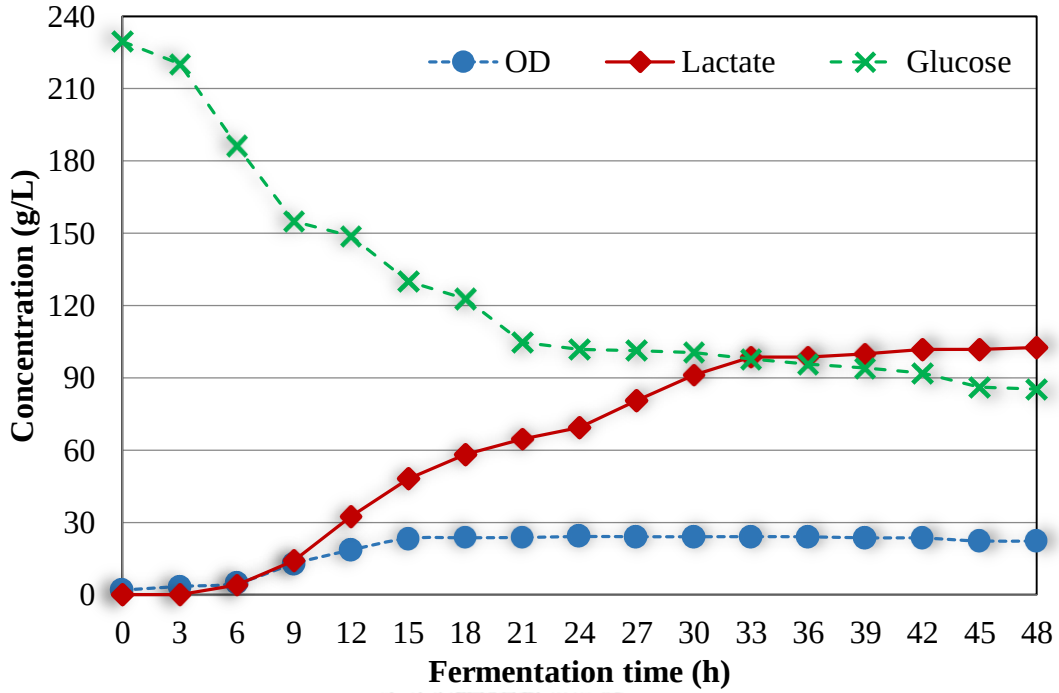


Figure 4.4 Fermentation profiles of *B. aerolacticus* BC-001 grown in glucose based medium at different glucose concentrations; 100 g/L (A), 125 g/L (B); 150 g/L (C); 175 g/L (D); and 200 g/L (E) in 5 L fermentor.

Table 4.3 Kinetics of batch fermentation with different initial glucose concentration effected on L-lactic acid production in 5 L fermentor

Initial glucose (g/L)	**Final lactate (g/L)**	**Yield (g/g)**	**Productivity (g/L·h)**	**Glucose uptake rate (g/L·h)**	**Remaining glucose (g/L)**	**Max OD_{600}**
100	104.24	0.85	5.79	6.77	0	30.40
125	106.38	0.91	3.94	4.33	27.70	30.40
150	121.01	0.85	3.10	3.64	34.70	29.57
175	118.94	0.90	2.48	2.75	66.22	28.20
200	102.51	0.71	2.14	3.00	85.43	24.07

4.3 Effect of different fed-batch fermentation on L-lactic acid production by *B. aerolacticus* BC-001

From the result of previously experiment (4.2), 100 g/L initial glucose was selected to to determine the proper fed-batch protocol. In this thesis, 3 fed-batch programs including intermittent feeding, constant mass flowrate feeding, and constant feed rate feeding were tested.

4.3.1 Intermittent fed-batch fermentation

Intermittent fed-batch fermentation was operated by adding 500 g/L glucose (50 mL) after batch operation was initiated for 6 h. The feeding was repeated every 3 h until 15 h (Figure 4.5). Feeding was stopped after 15 h due to the fermentor reached the maximum working volume. After 39 h, it was found that operating the fermentor using

the intermittent feeding program produced 145.08 g/L L-lactic acid with the corresponding yield of 0.95 and the overall productivity of 3.72 g/L·h. At the end of fermentation (42 h) when lactate production stopped, the glucose concentration of 14.65 g/L was remained in the fermentation broth.

From Figure 4.5 it was observed that from 3 to 12 h, glucose uptake rate was rather rapid while the production rate slightly decreased later. It was found that high lactate productivity was rather achieved at 6 h when the remaining glucose was approximately 50-60 g/L. At the glucose concentration lower than that, low productivity was obtained. This somewhat indicated the insufficient feeding of glucose. Therefore, to be able to maintain the remaining glucose concentration at the proper level, other feeding programs were introduced in fed-batch operation for lactate fermentation by *B. aerolacticus* BC-001.

4.3.2 Constant mass flow rate fed-batch fermentation

Concentrated glucose solution (500 g/L) was constantly fed at the mass flowrate of 10 g/L·h into the stirred fermentor initially operated by batch cultivation for 6 h. The mass flowrate was precalculated using the kinetics data obtained from the intermittent fed-batch operation at the highest lactate productivity during 3-12 h (4.3.1 and Figure 4.5). Detailed calculation can be found elsewhere (Appendix B). Similarly to intermittent feeding program, feeding was stopped after 15 h when the fermentor reached its maximum working volume.

From Figure 4.6, it was found that using constant mass flowrate could produce final lactate concentration up to 144.52 g/L similarly to that obtained from intermittent feeding program. Lactate yield of 1.09 g/g and the overall productivity of 3.44 g/L·h

were obtained. At the end of fermentation (45 h), very high glucose concentration up to 78.69 g/L was remained. Unlike that expected, feeding glucose at the maximum glucose uptake rate of 10 g/L·h to be able to maintain optimal glucose level in the fermentation broth was excess. It was suggested to reduce the mass flow rate in order to optimize the operation so that the desirable glucose level could be maintained.

4.3.3 Constant feed rate fed-batch fermentation

Constant feed rate fed-batch program is considered as the simplest feeding operation since it is easy to operate, utilizes less manpower compared to other feeding programs. In this thesis, this feeding program was tested for its possibility to apply in fed-batch fermentation by *B. aerolacticus* BC-001 for lactate production. To begin fed-batch operation using constant feed rate program, the feed rate predetermined elsewhere (Appendix B) was set at 45 mL/h. The concentrated glucose solution (500 g/L) was fed into the batch culture previously initiated for 6 h. The feeding was continued until 21 h when the fermentation reached the maximum working volume.

From Figure 4.7, constant feed rate program yielded the final lactate concentration of 103.43 g/L which was lowest compared to the other 2 feeding programs studied. The lowest yield and productivity of 0.88 g/g and 2.46 g/L·h were obtained. At the end of the run, the remaining glucose still remained high at the concentration of 112.28 g/L. The level was even higher than lactate product obtained. It was also observed that after feeding was stopped at 21 h, the glucose concentration was reached 124.21 g/L with the low uptake rate after that. As a result, it seemed that constant feed rate might not be proper in fed-batch fermentation

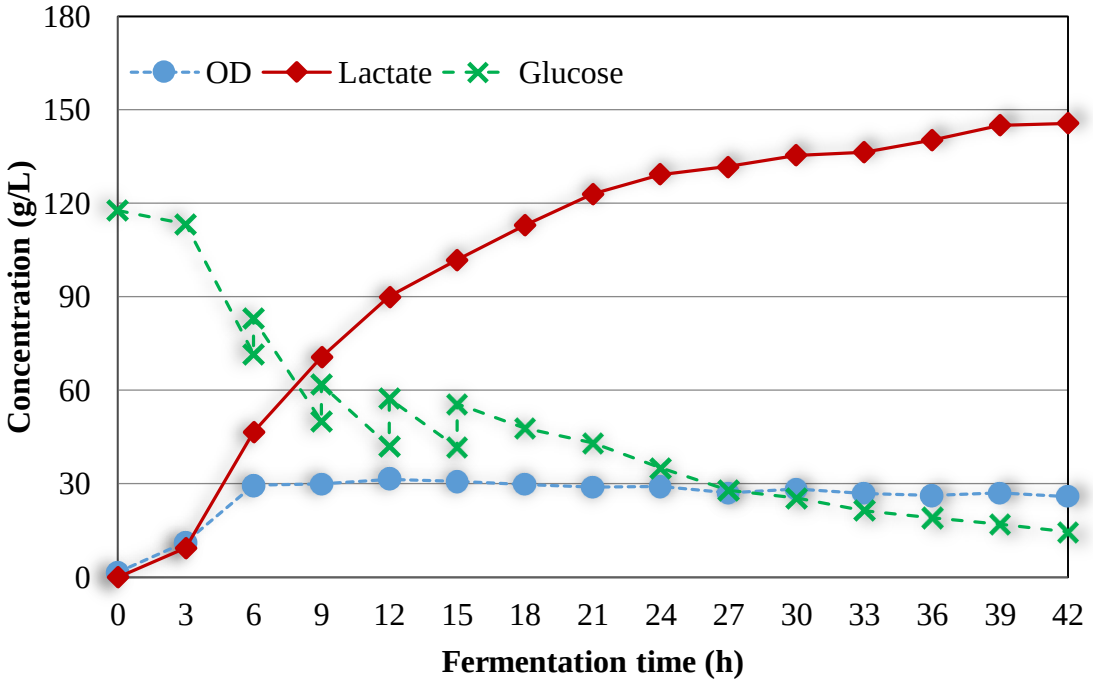


Figure 4.5 Intermittent fed-batch fermentation in 5 L stirred fermentor by *B. aerolacticus* BC-001 with 100 g/L initial glucose concentration

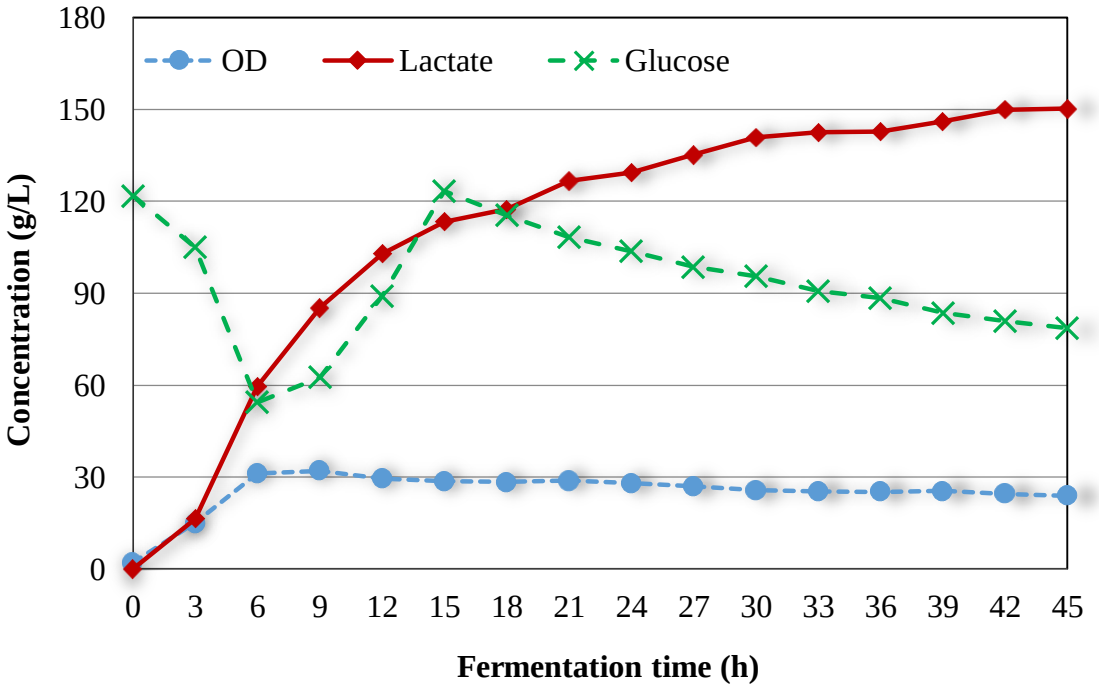


Figure 4.6 Constant mass flow rate fed-batch fermentation in 5 L stirred fermentor by *B. aerolacticus* BC-001 with 100 g/L initial glucose

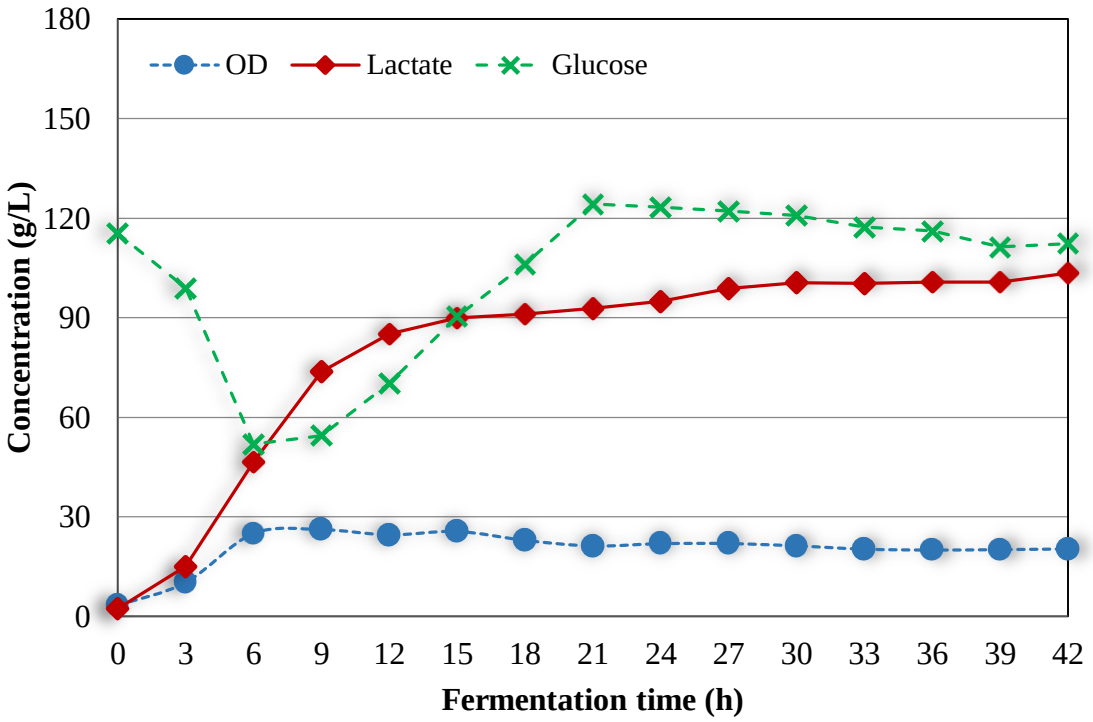


Figure 4.7 Constant feed rate fed-batch fermentation in 5 L stirred fermentor by *B. aerolacticus* BC-001 with 100 g/L initial glucose

4.3.4 Selecting the proper feeding program for fed-batch fermentation

The results present in Figures 4.5-4.7 indicate that lactate was rapidly produced during batch operation and the beginning of fed-batch culture. Later, the production rate was decreased in regardless of the feeding programs tested. Strong evidence of low productivity was shown when comparing the kinetics data of the 3 fed-batch operations tested with the batch cultivation (Table 4.4). It was found that the productivity was dropped by 35%, 41%, and 58% when applying intermittent, constant mass flowrate, and constant feed rate program during fed-batch culture compared with that from batch fermentation although the final lactate titer was increased by 37% when operating intermittent and constant mass flowrate fed-batch fermentation.

Among 3 feeding program, constant feed rate yielded the lowest lactate production that was even lower than that obtained from batch operation. In addition, the highest residual glucose remained at the end of fermentation leading to difficulty in harvesting and wastewater treatment. Therefore, this feeding program was dropped out for further study in this thesis. Although constant mass flowrate resulted in high lactate yield with the productivity comparable to intermittent feeding program, the unwanted residual glucose was still existed at the end of fermentation. Therefore, it was presumably concluded that intermittent feeding program was appropriate for fed-batch cultivation of *B. aerolacticus* BC-001 for L-lactic acid production. Regarding to the results by Qin et al (2009), 3 fed-batch fermentation were studied, e.g., pulse fed-batch strategy, constant residual glucose concentration fed-batch strategy, and exponential fed-batch strategy, pulse fed-batch strategy was the most effective strategy. By this

feeding program 150 g/L final lactic acid with 3.2 g/L·h was obtained by *Bacillus* sp. 2-6 (Qin et al., 2009).

Cell biomass production was proceeded at the early stage of fermentation and biomass concentration remained constant after 6 h. During fed-batch operation (after 6 h), biomass production was very low. This phenomenon indicated unfavorable condition during fed-batch operation. Since lactate is recognized as the growth associated product, anaerobic growth is mandatory to pursue for lactate production. It should be noted that most of lactic acid producing bacteria were fastidious. A wide range of growth factors including specific minerals, amino acids, vitamins, fatty acids, purines, and pyrimidines are required for their growth and biological activity. Several studies on nutrient requirement suggested that nitrogen source is necessary in lactic acid fermentation. The more the nitrogen source was to provide, the higher lactate concentration was obtained (Kwon et al., 2000; Nancib et al., 2005; Nancib et al., 2001). Among many nitrogen sources tested, yeast extract was reported as the simple nitrogen source used in lactate fermentation because of its excellent performance in promoting biomass and lactic acid production. However, using high concentration of yeast extract was not economically feasible in industrial production during high price (Gao and Ho, 2013).

As aforementioned, to further increase final lactate titer and to obtain sufficiently high productivity comparable to that obtained during batch culture, feeding supplemented nitrogen with glucose during fed-batch operation would be conducted. Ammonium salts including NH_4Cl and $(NH_4)_2SO_4$ were used in the feeding solution instead of using costly yeast extract.

Table 4.4 Fermentation kinetics of B. aerolacticus BC-001 during batch and fed-batch operations in the 5 L stirred fermentor.

Operation mode	**Batch**	**Fed-batch**		
		Intermittent feeding	**Constant mass flow rate**	**Constant feed rate**
Fermentation time (h)	18	39	42	42
Final lactate (g/L)	105.28	145.08	144.52	103.43
Productivity (g/L·h)	5.79	3.72	3.44	2.46
Yield (g/g)	0.85	0.95	1.09	0.88
Remaining glucose (g/L)	0	14.65	82.86	112.28
Max. OD_{600}	30.40 (at 9 h)	31.47 (at 12 h)	30.47 (at 9 h)	26.17 (at 9 h)

4.4 Effects of nitrogen source and C/N ratio on L-lactic acid production during fed-batch cultivation using intermittent feeding

Similary to 4.3.1, 50 mL concentrated glucose solution supplemented with NH_4Cl or $(NH_4)_2SO_4$ at different C/N ratios was intermittently fed into the 5 L stirred fermentor after the batch operation was initiated for 6 h. The feeding interval was set every 3 h until 18 h.

Starting with the low C/N ratio of 20:1, it was found that when adding both NH_4Cl and $(NH_4)_2SO_4$ into the concentrated glucose feeding solution, slight increase in final lactate titer with the similar biomass production was observed compared to the fed-batch culture fed with sole concentrated glucose solution (Table 4.5). Nevertheless, chloride ion is generally considered as toxic in many lactic acid producing bacteria when exposed at relatively high concentration (Roeßler et al., 2003). To avoid this fact, further C/N ratio optimization was conducted using only $(NH_4)_2SO_4$.

In case of feeding with concentrated glucose supplemented with $(NH_4)_2SO_4$, the C/N ratio of 40:1 gave the highest lactate production. Increasing C/N ratio from 20:1 to 40:1 resulted in increasing final lactate titer, yield, and productivity. Further increasing C/N ratio from 40:1 on the other hand resulted in lower lactate production. The proper C/N ratio determined in this study indicated the balanced glucose flux towards cell biomass, lactate, and maintenance energy production. As a result, this promoted growth associated product kinetics model and eventually increasing lactate production (Meng et al., 2012). This were also confirmed by Gao and Ho (2013). It was found that adding 0.5 g/L $(NH_4)_2SO_4$ could increase cell growth and lactic acid production but no change in both of biomass and final lactic acid when increasing $(NH_4)_2SO_4$ to 1-2 g/L when glucose was exhausted. It has been reported that ammonium ions can be harmful to the

cell cause of alkaline property that reduce lactic acid production capability. Moreover, the amount of excess nitrogen can inhibit the growth and lactic acid production as well (Leejeerajumnean et al., 2000).

Besides intermittent feeding, the concentrated glucose supplemented with $(NH_4)_2SO_4$ at the C/N ratio of 40:1 was fed into the fermentor using the other 2 different feeding programs (Figure 4.8). It was found that intermittent feeding program was still the suitable feeding program in lactate production (Table 4.6). Although nitrogen source was supplied in the feeding solution in order to promote growth associated product kinetics model, overall lactate productivity in fed-batch fermentation was still lower than that obtained from batch cultivation. Also, it was found that the highest productivity of 11.67 g/L·h was achieved during 3-9 h but later it was dropped. Therefore, it is believed that cell physiology during fermentation might play role on metabolic response to environmental condition. To observe the physiological stage of *B. aerolacticus* BC-001, samples during fermentation were taken for scanning electron microscopy (Figure 4.9). From the cell physiology, it is clear that the high productivity was obtained during cell elongation period. Therefore, it is suggested that high productivity could be obtained if the sufficient elongated cell numbers were maintained throughout the fermentation run.

Table 4.5 Effect of nitrogen source on L-lactic acid fermentation by *B. aerolacticus* BC-001 during intermittent fed-batch fermentation in the 5 L stirred fermentor

Feeding solution	**C/N ratio**	**Time (h)**	**Final lactate (g/L)**	**Glucose consumed (g/L)**	**Productivity (g/L·h)**	**Yield (g/g)**	**max OD_{600}**
Glucose	-	39	145.08	152.66	3.72	0.95	31.47 (at 12 h)
Glucose with NH_4Cl	20:1	45	149.12	156.89	3.31	0.95	33.03 (at 15 h)
Glucose with $(NH_4)_2SO_4$	20:1	45	151.86	159.61	3.37	0.95	31.73 (at 15 h)
	40:1	45	160.84	157.67	3.57	1.02	32.67 (at 18 h)
	60:1	45	151.96	161.61	3.38	0.94	32.90 (at 9 h)
	80:1	45	148.43	171.17	3.30	0.87	32.67 (at 9 h)

Table 4.6 Effect of glucose solution mixed with $(NH_4)_2SO_4$ at C/N ratio 40:1 on different fed-batch fermentation on l-lactic acid production by *B. aerolacticus* BC-001

Kinetics	**Intermittent**	**Constant mass flow rate**	**Constant feed rate**
Fermentation time (h)	45	45	45
Final lactate (g/L)	160.84	136.60	111.41
Productivity (g/L·h)	3.57	3.04	2.48
Yield (g/g)	1.02	0.96	0.97
Remaining glucose (g/L)	17.74	63.79	81.21
Max. OD_{600}	32.67 (at 18 h)	32.17 (at 18 h)	27.03 (at 12 h)

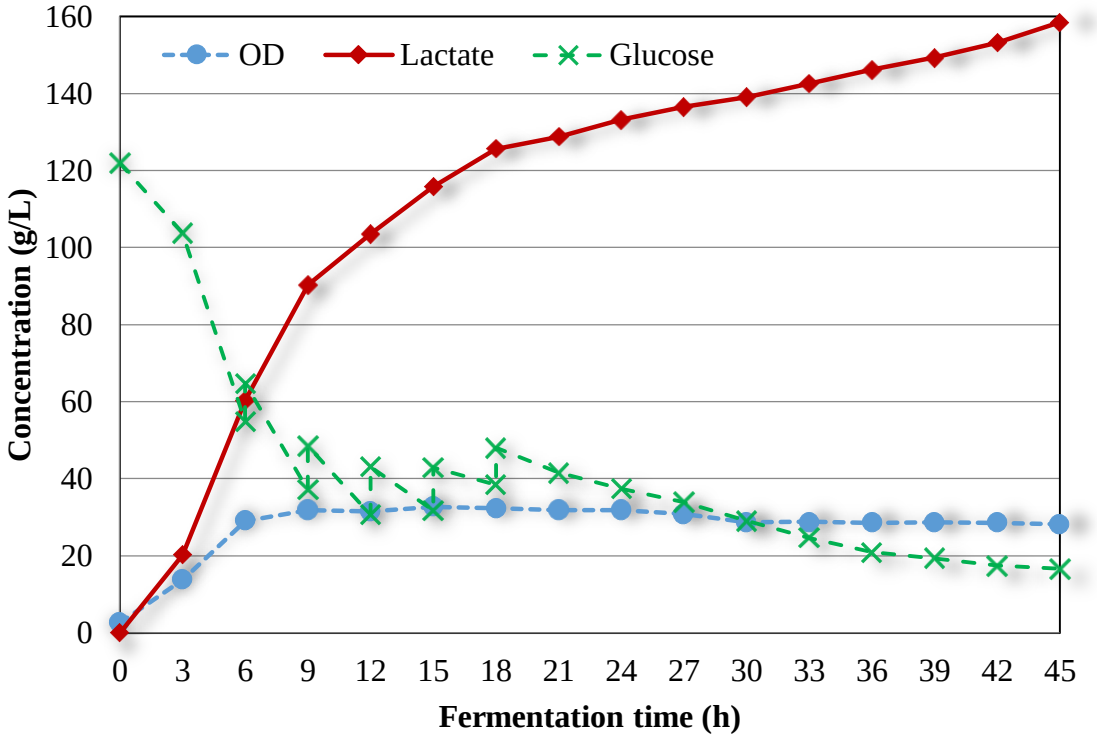
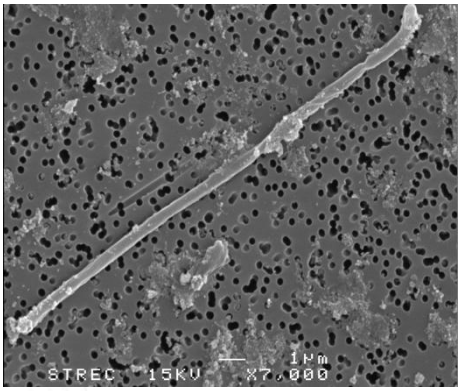
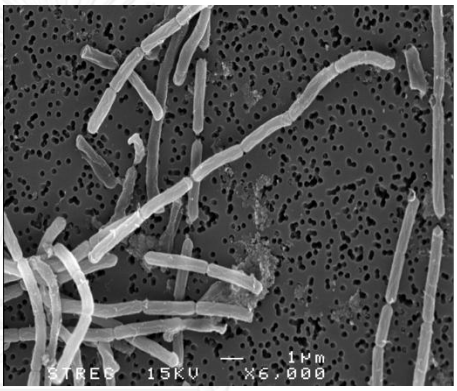
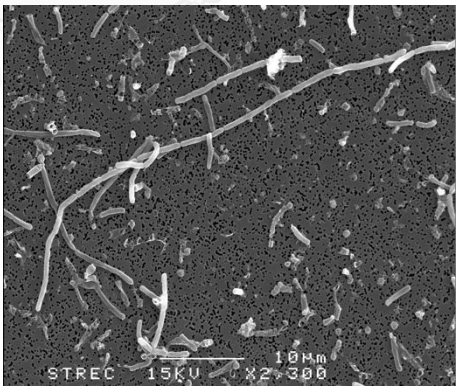


Figure 4.8 Effect of glucose solution mixed with $(NH_4)_2SO_4$ at C/N ratio 40:1 on intermittent fed-batch fermentation on L-lactic acid production by *B. aerolacticus* BC-001

Fermentation time (h)	Intermittent Fed-batch fermentation
0	
3	
6	

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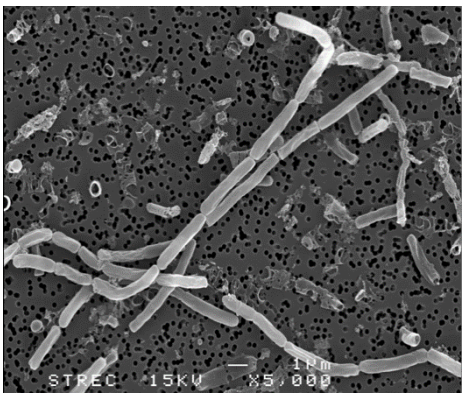
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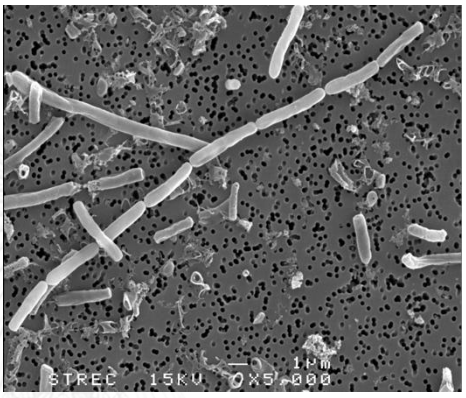
12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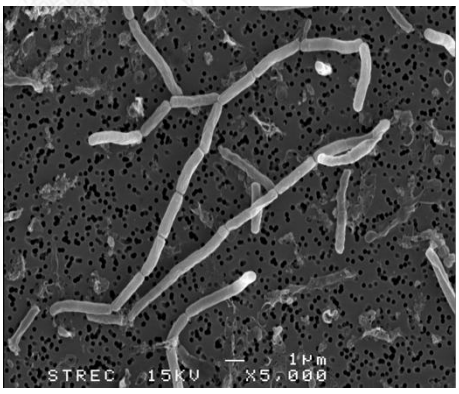
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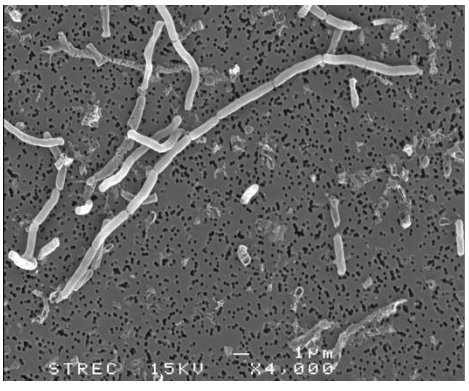
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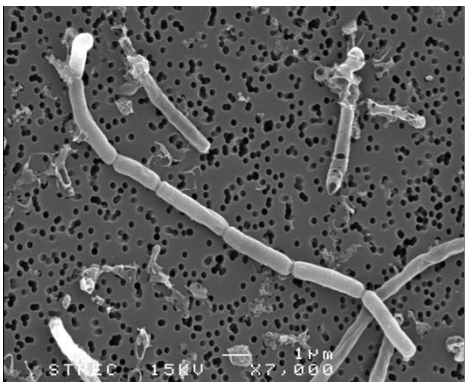
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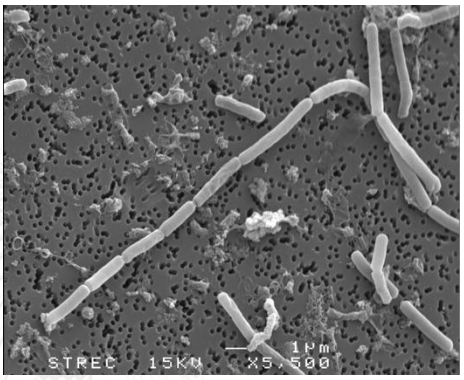
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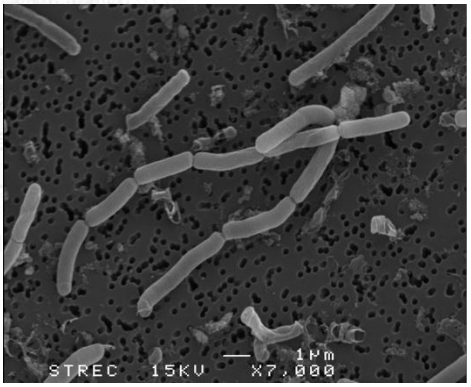
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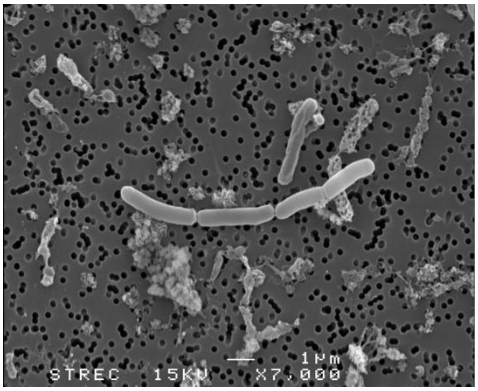
30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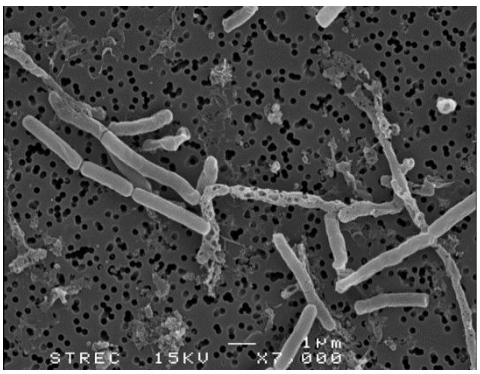

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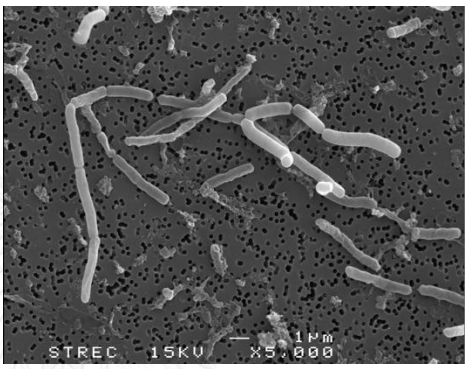

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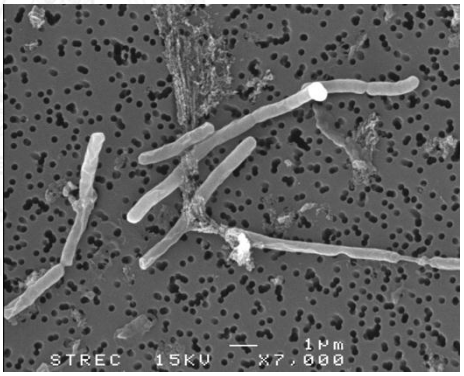

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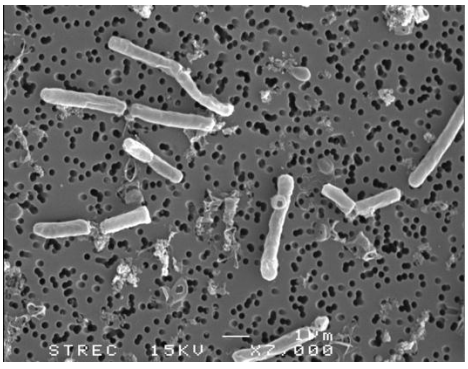

Figure 4.9 Scanning electron micrographs of *B. aerolacticus* BC-001 during cultivation in intermittent Fed-batch fermentation

CHAPTER 5

CONCLUSION AND RECOMMENDATIONS

5.1 Summary

B. aerolacticus BC-001 was proven as the potential industrial strain for L-lactic acid fermentation. Not only solely producing an optically pure L-lactic acid which is essential in PLA synthesis, but it was also found that this isolate could also grow and ferment lactic acid at the acceptably high yield and productivity. Further increasing final lactate titer up to 192 g/L with the acceptable yield of 0.90 g/g and productivity of 3.55 g/L·h from fermentation by this isolate could be achieved by simple intermittent fed-batch fermentation in shake flask.

However, when scale up to the 5 L stirred fermentor, the high final lactate titer was not achieved in the similar way to that in shake flask. In typical batch fermentation with sole glucose solution in the 5 L fermentor, *B. aerolacticus* BC-001 produced 104.24 g/L lactate titer with the productivity and yield of 5.79 g/L·h and 0.85 g/g respectively. When increasing the initial glucose concentration to 125-200 g/L, substrate repression was appeared. By this, different fed-batch fermentation was conducted using 3 different feeding programs including intermittent fed-batch fermentation, constant mass flow rate fed-batch fermentation, and constant feed rate fed-batch fermentation. The most effective strategy was intermittent fed-batch fermentation, with sole glucose solution, 145.08 g/L final lactate concentration with productivity of 3.72 g/L·h and yield of 0.95 g/g were obtained. For constant mass flow

rate fed-batch fermentation and constant feed rate fed-batch fermentation, due to over feeding, excess residual glucose was appeared at the end of fermentation. Moreover, it was found that when adding glucose solution supplemented with $(NH_4)_2SO_4$ at the C/N ratio of 40:1 was able to improve final lactate titer to 160.84 g/L with 3.58 g/L·h productivity and 1.02 g/g yield. Therefore, compared with the typical batch fermentation, intermittent fed-batch fermentation with glucose solution supplemented with $(NH_4)_2SO_4$ at the C/N ratio 40:1 yielded the increasing final lactate concentration for 54.30%, yield for 11.76%, and being able to prolong the correct physiological stage for lactate formation. Therefore, the high productivity zone of 11.70 g/L·h was extened until 18 h of fermentation. Moreover, the medium composition cost for L-lactic acid production (160 g/L) by batch fermentation was higher than intermittent fed-batch fermentation for 25%. By the way, intermittent fed-batch fermentation with glucose solution supplemented with $(NH_4)_2SO_4$ at the C/N ratio 40:1 was not prolong high productivity until the end of fermentation because only carbon and nitrogen sources were not sufficient to maintain the cell elongation period.

5.2 Recommendations

In our experiment, it was found that lactic acid production by *B. aerolacticus* BC-001 gradually dropped after 18 h fermentation. The results from the scanning electron micrographs show that when *B. aerolacticus* BC-001 had a high productivity (0-18 h), cell was elongated and divided. This was presumably due to the limitation of some nutrients which was required in cell growth and maintenance activity of *B. aerolacticus* BC-001.

A number of study about adding nutrient supplementation to improve lactic acid production suggest to add glutamine; a good source of nitrogen for most bacteria, and can be converted to glutamate, the major donor of nitrogen for amino acid and nucleotide biosynthesis (Gao and Ho, 2013; Hu et al., 1999) or $(NH_4)_2SO_4$ supplemented with B vitamin complex to enhancing effect on lactic acid production (Nancib et al., 2005). Therefore, one should take into consideration that some amino acid or vitamin could be supplemented with glucose during long-term cultivation in order to maintain fermentor productivity.

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APPENDIX A

ANALYTICAL METHOD

A1 High Performance Liquid Chromatography

High performance liquid chromatography (HPLC) was used to analyze the organic compounds (glucose, lactic acid, fumaric acid, and ethanol) present in the fermentation broth. Samples from this studies were diluted with double distilled water (DDI water). After that diluted particle-free sample (15 µL) were injected into an organic acid analysis column (Biorad, Aminex HPX-87H ion exclusion organic acid column; 300mm x 7.8mm) maintained at 45°C in a column oven (Shimadzu-CTO-6A). 0.005 N H_2SO_4 was used as an eluent at 0.6 mL/min flow rate. An RI detector (Shimadzu-RID-6A) was set at the range of 200 to detect the organic compounds. A standard containing 2 g/L of each component was injected as a reference to determine the sample concentration in rage of 0 to 2 g/L (preparation follow below).

Concentration (g/L)	Standard 2 g/L (µl)	DDI water (µl)
0.25	125	875
0.5	250	750
1.0	500	500
1.5	750	250
2.0	1000	-

Retention time of glucose, lactic acid, fumaric acid, and ethanol is 9.525, 13.386, 15.117, and 22.442 respectively.

A2 Glucose Analyzer

Glucose and L(+)-lactic acid were analyzed by YSI 7100 glucose analyzer (Yellow Spring Instrument Co., Inc.). This analytical instrument is accurate within the range of 0-2 g/L glucose and L-lactic acid. Before measurement, fermentation broth was centrifuged and diluted with distilled water.

The calibrator standard contained

L(+)-lactic acid	0.5 g/L
glucose	2.5 g/L
benzoic acid	1.0 g/L
NaEDTA	2.0 g/L

The buffer powder used in this equipment was prepared by mixing

K_2H_2EDTA	4.4 g
kanamycin sulfate	0.05 g
sodium benzoate	7.3 g
NaH_2PO_4	12.0 g
Na_2PO_4	54.7 g
NaCl	21.5 g

The buffer solution was prepared by dissolving 12.7 g buffer powder in 900 mL distilled water.

APPENDIX B

CALCULATION

1. Yield (g/g)

$$\text{Yield} = \frac{\text{(Final lactic acid (g/L))}}{\text{(Initial glucose – Remaining glucose (g/L))}}$$

2. Productivity (g/L·h)

$$\text{Productivity} = \frac{\text{(Final lactic acid (g/L))}}{\text{(Fermentation time (h))}}$$

3. Glucose uptake rate (g/L·h)

$$\text{Glucose uptake rate} = \frac{\text{(Glucose accumulation (g/L))}}{\text{(Fermentation time (h))}}$$

4. Feed rate (g/L·h)

4.1 Constant mass flow rate fed-batch fermentation

Substrate concentration in fermentor	=	**Feeding Substrate concentration**	-	**Diluted Substrate concentration**	+	**Used substrate concentration (for cell maintenance and LA production)**
$\frac{dS}{dt}$	=	$\frac{F}{V}Sf$	-	$\frac{F}{V}Si$	+	r
$\frac{dS}{dt}$	=	$\frac{F(Sf-Si)}{V}$	-		+	r

Where;

F = Feeding rate (g/L·h)

Sf = Substrate feeding stock concentration (g/L)

Si = Residual substrate concentration in fermentor (g/L)

V = Broth volume (L)

t = Time (h)

r = Substrate uptake rate (g/L·h)

To maintain substrate in fermentor in steady, $\frac{dS}{dt} = 0$

$$\frac{F(Sf-Si)}{V} + r = 0$$

$$F = \frac{rV}{(Sf-Si)}$$

For example, profile from intermittent fed-batch fermentation (Figure 4.5) show that 3-12 h of fermentation had high productivity. Hence, glucose uptake rate was calculated to use feed sufficiently glucose.

Table B-1 Kinetic of intermittent fed-batch fermentation

Fermentation time (h)	**OD_{600}**	**Lactate (g/L)**	**Glucose accumulation (g/L)**
0	1.55	0	0
3	11.23	9.37	4.42
6	29.47	46.72	41.58
9	29.93	70.59	32.73
12	31.47	90.16	19.72
15	30.70	101.82	15.62
18	29.83	113.05	7.75

Glucose uptake rate = $\frac{\text{(Glucose accumulation (g/L))}}{\text{(Fermentation time (h))}}$

3-12 h

= $\frac{\text{(4.42 + 41.58 + 32.73 + 19.72 (g/L))}}{\text{(12 - 3 h)}}$

= 10.94 g/L·h

From calculation, approximate 10 g/Lh was used for cell maintenance and lactic acid production. By this, 10 g/L·h is required to feed into the fermentor.

$$F = \frac{rV}{(Sf - Si)}$$

Where;

F	=	Feeding rate at 6 h		
r	=	Glucose uptake rate (g/L·h)	=	10 g/L·h
V	=	Broth volume at 6 h	=	2 L
Sf	=	Substrate feeding stock concentration (g/L)	=	500 g/L
Si at 6 h	=	Residual substrate concentration in fermentor at 6 h (g/L)	=	71.77 g/L

Table B-2 Feed rate calculation

Fermentation time (h)	Time (h)	Volume (L)	Feeding rate (L/h)	Feeding rate(mL/h)
6	0	2	0.047	46.70
7	1	2.047	0.048	47.79
8	2	2.094	0.049	48.91
9	3	2.143	0.050	50.05
10	4	2.193	0.051	51.22
11	5	2.245	0.052	52.42
12	6	2.297	0.054	53.64
13	7	2.351	0.055	54.89
14	8	2.406	0.056	56.18
15	9	2.462	0.057	57.49
16	10	2.519	0.059	58.83
17	11	2.578	0.060	60.20
18	12	2.638	0.062	61.61

After calculation, pump was set up to conduct 500 g/L glucose solution stock into fermentor.

4.2 Constant feed rate fed-batch fermentation

The same feed rate at 6 h of constant mass flow rate was used.

5. C/N ration

Basic information of glucose:

Molecular weight = 180.156 g/mole

Carbon 6 atoms = 6 x 12.011 = 72.066 g

Glucose 500 g/L:

Glucose	180.156 g	have	Carbon	72.066 g
Glucose	500 g	have	Carbon	200.01 g

C/N ratio = 20:

Gram of nitrogen = Gram of Carbon / 20

= 200.01 g / 20

= 10.001 g

Use NH_4Cl as nitrogen source

(MW = 53.492 g/mole, nitrogen = 14.007 g/mole)

Nitrogen	14.007 g	was composed in	NH_4Cl	53.492 g
Nitrogen	10.001 g	was composed in	NH_4Cl	38.193 g

∴ 1 L stock solution of C/N 20:1 consist of 500 g glucose + 38.193 g NH_4Cl

Use $(NH_4)_2SO_4$ as nitrogen source

(MW = 132.139 g/mole, nitrogen 2 atom = 28.014 g)

Nitrogen	28.014 g	was composed in	$(NH_4)_2SO_4$	132.139 g
Nitrogen	10.001 g	was composed in	$(NH_4)_2SO_4$	47.174 g

∴ 1 L stock solution of C/N 20:1 consist of 500 g glucose + 47.174 g $(NH_4)_2SO_4$

For C/N 40:1, 60:1, and 80:1, calculate the same way of C/N 20:1.

VITA

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Research presentation experience

Wutthimongkholchai, A., Piluk, J., Thitiprasert, S., Tolieng, V., Prasitchoke, P., Thongchul, N. A novel isolate Bacillus sp. BC-001 and its industrial potential in producing L-lactic acid with high titer. Poster presentation and proceedings. The 5th International Biochemistry and Molecular Biology Conference 2016 "BMB 2016". 26-27 May 2016. B.P. Samila Beach hotel, Songkhla, Thailand.